UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS

New microbial inocula for bioaugmentation: novel product design and valorization

"Documento Definitivo"

Doutoramento em Biologia
Especialidade de Microbiologia

Pedro Jorge Dias Silvestre Teixeira

Tese orientada por:
Professor Doutor Rogério Paulo de Andrade Tenreiro
Doutora Sandra Isabel Mourinha Lopes Chaves

Documento especialmente elaborado para a obtenção do grau de doutor

2020

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Bolsa de doutoramento em empresa SFRH/BDE/95903/2013, pela Fundação para a Ciência e a Tecnologia e empresa BioTask, Biotecnologia, Lda

2020

Agradecimentos

Este trabalho científico representa o final de uma determinante etapa de crescimento profissional e pessoal difícil de descrever. Os anos aqui investidos foram resultado de perseverança, trabalho e determinação pessoal, mas não o fiz sozinho. Este trabalho não teria sido possível sem a presença de pessoas excecionais, que me apoiaram e ajudaram.

Gostaria de agradecer ao Professor Doutor Rogério Tenreiro, responsável pelo grupo de Microbiologia e Biotecnologia do BioISI (M&B – BioISI), pela sua inestimável orientação científica e apoio. Não vou esquecer a postura positiva, sempre disponível a ajudar, com críticas construtivas e conselhos, sempre exigente e rigoroso em todas as áreas.

À Professora Doutora Ana Tenreiro, obrigado pela simpatia, amabilidade e boa disposição que traz ao laboratório. Agradeço também à Professora Doutora Manuela Carolino, pela oportunidade de partilhar parte do conhecimento adquirido sobre biorremediação e áreas afins, no módulo de Laboratório em Microbiologia, do Mestrado de Microbiologia Aplicada (FCUL). Ao professor Doutor Carlos Cordeiro, responsável pelo Grupo de Enzimologia do Centro de Química e Bioquímica da Faculdade de Ciências da Universidade de Lisboa, obrigado pela ajuda nos métodos de análise por HPLC. À *Chief Laboratory Office* do laboratório de M&B – BioISI, Filipa Silva, agradeço por toda a ajuda durante o tempo passado no laboratório.

A todos com quem dividi o espaço laboratorial, em especial a Ana Marta, a Ana Soares, ao André, a Beatriz, a Carolina, a Catarina, a Claúdia, a Inês, ao João, a Mariana, obrigado pelas horas de conversa e discussão, pela companhia nas horas de almoço ou apenas pelo "*Bom Dia*" pela manhã. São estes momentos que nos fazem manter a sanidade mental. Um agradecimento especial a Cláudia Luís, pelas conversas e acesas discussões durante o tempo que trabalhámos em conjunto.

Gostaria também de agradecer ao Dr. Vitor Sérgio Silva e Eng.º Paulo Bastos, gerentes da empresa *BioTask, Biotecnologia, Lda*, pelo apoio e confiança depositada ao longo destes anos. Trarei para sempre os conselhos partilhados, que me fizeram crescer profissional e pessoalmente. De facto, a sorte dá muito trabalho a encontrar!

Aos colegas da *BioTask*, o Pedro, o Tiago, o Fernando, a Margarida e o Manuel, obrigado pelo apoio, pelos momentos partilhados, as reuniões da empresa e todas as conversas pelo meio.

Aos meus pais e ao meu irmão, obrigado pelo apoio durante todos estes anos, pela paciência e pela ajuda, em tudo quanto precisei. Sei que posso contar convosco sempre, e estarei aqui para vocês também.

Aos amigos de casa, vocês sabem quem são, o que crescemos durante este tempo. Alguns casámos, tivemos filhos e continuamos todos juntos! Apenas espero que continuem por cá, são a família que eu escolhi.

O último agradecimento deixo a quem mais me ajudou, de forma incondicional, quem me ouviu durante todo este tempo, mesmo quando eu podia não merecer essa paciência. Quem soube, também, guiar-me durante este período e mostrar-me o que é verdadeiramente importante na vida. Tu és a minha família. Obrigado por seres quem és, Carla Sofia Guilherme Silvestre.

Para ti, Mateus Silvestre Teixeira

Resumo

A biorremediação é o processo que utiliza microrganismos ou seus subprodutos para degradar e descontaminar resíduos e/ou poluentes de solos, água ou ar. Podem ser adicionados microrganismos viáveis ou seus subprodutos, ou recorrer à estimulação da microbiota naturalmente presente, por processos químicos (correção de pH, adição de nutrientes) ou mecânicos (homogeneização, arejamento ou outros). Estes conceitos, definidos quando se estabeleceu a biotecnologia ambiental, estão desfasados da prática corrente no mercado. No entanto, existem algumas empresas que disponibilizam produtos à base de microrganismos (na grande maioria bactérias) para promover a bioaumentação como forma de tratamento de efluentes altamente contaminados. A bioaumentação baseia-se na adição de culturas de microrganismos viáveis que contenham as vias metabólicas necessárias para a degradação dos principais contaminantes poluentes de uma massa de água ou solo. A grande maioria destes produtos comerciais têm por base estirpes dos géneros *Bacillus* e *Pseudomonas*, com poucos resultados científicos publicados, quando aplicados à escala real. No entanto, o avanço nas metodologias de multiplicação e produção de microrganismos tem permitido reduzir custos e atingir outras áreas, como a biotecnologia ambiental. Paralelamente, as novas tecnologias de sequenciação (de terceira e quarta geração) vêm permitir estudar sistemas biológicos altamente complexos e dinâmicos, como os que ocorrem em Estações de Tratamento de Águas Residuais (ETARes), ou mesmo solos contaminados. Estes fatores potenciam a biotecnologia ambiental e o estabelecimento de novos produtos e ferramentas que promovam a biodegradação dos poluentes produzidos pela sociedade atual.

Este trabalho teve por objetivo isolar, caracterizar e selecionar microrganismos de origem natural, que possam ser aplicados à escala real, com potencial para integração em novos produtos de bioaumentação. Para o isolamento de microrganismos com capacidades degradativas, foram adotadas duas estratégias distintas, por isolamento direto de amostra ambientais altamente contaminadas e isolamento seletivo após ensaios de evolução adaptativa, terminando numa comparação entre os isolados selecionados e produtos comerciais já disponíveis no mercado, em ensaios de biodegradação em condições laboratoriais controladas.

Do decorrer da primeira estratégia, procedeu-se ao isolamento direto a partir de 9 amostras ambientais, de locais distintos e normalmente expostos a grande concentração de poluentes, como as ETARes. Destes locais foram obtidos 166 isolados bacterianos, para caracterização e rastreio de capacidades lipolíticas. Foram também analisados produtos comerciais já disponíveis no mercado, obtendo-se 30 isolados bacterianos, que serviram de *benchmark* para

avaliação dos isolados de origem natural. Este conjunto de 196 microrganismos foi caracterizado para a degradação de óleos e gorduras (FOG) em ensaio de microplaca, utilizando o meio mineral M9 suplementado com uma única fonte de carbono. Utilizaram-se lípidos como fonte de carbono, o ácido oleico (um ácido gordo livre monoinsaturado) e a trioleína (um triglicérido, contendo três resíduos de ácido oleico ligados a uma molécula de glicerol), em duas concentrações. A caracterização fenotípica sumária (morfologia celular, coloração de Gram, oxidase e catalase), bem como a análise de perfis genómicos, obtidos por PCR-fingerprinting recorrendo aos primers csM13 e PH, permitiu estudar a diversidade destes isolados e definir grandes grupos de maior interesse. A abordagem integrativa por Análise de Componentes Principais permitiu selecionar 9 microrganismos de elevado potencial para biorremediação de efluentes com alto teor em FOG, com base na área debaixo da curva obtida pelos ensaios em microplaca. Os isolados selecionados foram identificados por sequenciação parcial do gene de rRNA 16S e aplicados em ensaios de biodegradação efetiva, tendo dois isolados, *Aeromonas* sp. BBC|043 e *Staphylococcus cohnii* BBC|148, revelado a melhor capacidade degradativa de FOG em termos de potencial de aplicação futura em produtos de bioaumentação.

Para a segunda estratégia, foram mantidos 4 ensaios de evolução adaptativa em laboratório, a partir de um inóculo ambiental misto, contendo como única fonte de carbono em cada ensaio um composto poluente insolúvel e recalcitrante. Durante o processo de evolução adaptativa, uma população microbiana é mantida durante vários ciclos em condições de stress, que promovam o estabelecimento de estirpes de subpopulações mais adaptadas. No caso de se utilizar um consórcio natural, espera-se que ocorra um "*wash-out*" de subpopulações que não intervêm diretamente nos processos de biodegradação, visto que serão nutricionalmente dependentes e minoritárias em relação às subpopulações degradadoras, podendo, no entanto, ocorrer processos de coevolução. Para as experiências de evolução adaptativa, as fontes de carbono testadas foram: antraceno e fenantreno (representantes de hidrocarbonetos aromáticos policíclicos, PAH), triestearina (triglicérido insaturado) e óleo mineral lubrificante (mistura de hidrocarbonetos alifáticos). Todos estes poluentes são insolúveis em água e de difícil biodegradação, tendo-se criado um meio de crescimento estratificado, com uma fase líquida, uma interface e o poluente não dissolvido. Este ensaio foi seguido durante mais de 2 anos, com 110 ciclos, tendo-se isolado 455 microrganismos (bactérias e leveduras) através de meios seletivos para os mesmos poluentes em todos os ensaios. Os isolamentos foram duplamente cruzados, tendo-se pesquisado degradadores dos quatro poluentes em cada um dos diferentes ensaios de evolução adaptativa. Estendendo a metodologia anterior, os isolados foram

caracterizados fenotipicamente e por fingerprinting genómico, aplicando os primers csM13, PH e $[GTG]_5$. A análise genómica permitiu selecionar as estirpes mais frequentes em todos os ensaios e potencialmente envolvidas na biodegradação dos poluentes, tendo-se identificado 19 microrganismos. Para compreender a dinâmica dos principais grupos microbianos durante o processo de evolução adaptativa, recorreu-se a métodos de sequenciação de 3ª geração, tanto para bactérias (sequenciação parcial do gene de rRNA 16S) como para fungos (sequenciação parcial do gene de rRNA 26S). Esta abordagem permitiu avaliar as alterações da diversidade relativa de diferentes grupos de bactérias e fungos ao longo do tempo e a influência dos substratos. Adicionalmente, permitiu verificar a presença de diferentes grupos de fungos, que não foram obtidos pelos métodos de isolamento, à exceção de leveduras do género *Yarrowia*. No entanto, ao nível das bactérias, ocorreu grande concordância dos géneros isolados (*Acinetobacter*, *Brevundimonas*, *Paraburkholderia*, *Pigmentiphaga*, *Pseudomonas* e *Sphingobacterium*) como sendo determinantes ao longo do processo de evolução adaptativa.

A fase final passou pelo desenvolvimento de métodos de quantificação dos compostos poluentes alvo, passíveis de aplicação à escala laboratorial, em volumes reduzidos e de análise relativamente rápida. No caso dos FOG, o método gravimétrico demonstrou ter capacidade e resolução suficiente para os ensaios de biodegradação. Esta é uma técnica recorrente em laboratórios de análises de efluentes, que apresenta alguma variabilidade entre laboratórios. No entanto, foi possível aferir a capacidade de mais de 20 isolados, em comparação direta com produtos comerciais já no mercado, com resultados de biodegradação bastante promissores para resíduos de amostras ambientais, oriundas de separadores de gordura. O melhor resultado foi atingido pelo isolado *Aeromonas* sp. BBC|043, capaz de remover perto de 90% de FOG em apenas 5 dias de ensaio, seguido do isolado BBC|650, que alcançou 55% de remoção. Neste ensaio, os produtos comerciais testados atingiram valores de redução inferiores (40%), o que demonstra o potencial de melhoria que estes novos isolados podem conferir, face aos existentes no mercado. Nos ensaios de biodegradação de PAH, foi testada a capacidade de remoção de antraceno e fenantreno, dois poluentes recalcitrantes, pelo conjunto de isolados previamente selecionado. Neste caso, foi possível definir um método de quantificação de PAH por cromatografia líquida de alta performance (*HPLC*). O ensaio de biodegradação permitiu demonstrar eficiência dos microrganismos selecionados, capazes de remover até 40% de antraceno e até 60% de fenantreno, em apenas 5 dias de ensaio. Os microrganismos mais promissores estão associados a géneros bacterianos já descritos, como *Pseudomonas* e *Paraburkholderia*, que contêm vias metabólicas já descritas para a degradação destes compostos. No entanto, dado os ensaios de evolução adaptativa, estas

novas estirpes poderão conter modificações de elevado interesse biotecnológico, que lhes confiram uma vantagem competitiva relativamente aos restantes, com maior eficiência ou afinidade para estes poluentes.

Os objetivos estabelecidos foram parcialmente cumpridos durante a execução do presente trabalho de doutoramento. Foi possível isolar microrganismos de elevada capacidade degradativa para remoção de FOG e PAH, a partir de duas estratégias de isolamento distintas. Foi possível caracterizar e selecionar microrganismos de elevado potencial para a degradação de diferentes poluentes, tendo sido possível determinar capacidades efetivas de remoção, em ensaios de biodegradação em laboratório. Um dos objetivos secundários definidos inicialmente passava por ensaios utilizando efluente real, simulando condições operacionais, que não foi possível executar. No entanto, os ensaios utilizando resíduos de separadores de gordura, em laboratório, demonstraram resultados muito promissores para os microrganismos selecionados.

Os resultados obtidos suportam claramente a aposta na biorremediação enquanto técnica para a eliminação de poluentes. Foi possível demonstrar a eficiência de produtos já existentes no mercado e a melhoria que estes novos isolados poderão introduzir no futuro. A biodegradação de PAHs, um tipo de poluente recalcitrante considerado como um dos poluentes emergentes a nível mundial, deve ser encarada como ferramenta viável para lidar de forma efetiva com este grave problema ambiental. Estes resultados promissores reforçam o interesse num setor com pouco investimento nacional, com a possibilidade de desenvolver novos produtos de alto valor acrescentado, num trabalho que incluiu financiamento direto de uma empresa portuguesa de biotecnologia ambiental, a *BioTask, Biotecnologia, Lda*.

Palavras-chave:

Biorremediação; Evolução adaptativa; PAH; FOG; Biodegradação

Abstract

Biological processes as bioremediation hold a promising alternative to overcome pollution accumulation worldwide. There are commercial products available for bioremediation, most based on strains from few species of *Bacillus* and *Pseudomonas*. In the present work, both direct isolation and adaptive evolution experiments (AEx) were set-up to screen new and improved microbial biodegraders towards fat, oils and grease (FOG) and polycyclic aromatic hydrocarbon (PAH) contaminants.

By the direct isolation, 196 microorganisms were screened and fingerprinted by PCR with primers csM13 and PH, allowing the selection of two bacterial strains, identified as *Aeromonas* sp. and *Staphylococcus* sp., with good biodegradation capacity for FOG residues. The AEx focused on PAH pollution and insoluble triglycerides, and were conducted for 110 cycles, creating a collection of 455 microorganisms (bacteria and yeast) by selective solid media isolation. All isolates were analysed by PCR-fingerprint with multiple primers (csM13, PH and $[GTG]_5$), to assess genomic diversity and detect indistinguishable isolates. The microbial population changes during AEx were also followed by third generation sequencing, to assess limitations and bias of the isolation strategy. A total of 19 microorganisms were selected and identified as belonging to *Acinetobacter*, *Brevundimonas*, *Paraburkholderia*, *Pigmentiphaga*, *Pseudomonas*, *Sphingobacterium* and *Yarrowia*, genera with known biodegradative ability. Methods were developed for effective pollutant removal quantification to compare the selected isolates. Concerning FOG residues, a gravimetric approach showed that the two best isolates removed 90% and 55% of FOG, after 5 days assay. For PAH, a HPLC quantification method revealed that the two best isolates were capable to remove 40% of anthracene and 60% phenanthrene, after 5 days assay.

These results pave the way for the integration of the selected strains into new products, with high added value and effectiveness as an ecological bioremediation alternative, to deal with recalcitrant pollutants as FOG and PAH.

Keywords:

Bioremediation; Adaptive evolution; PAH; FOG; Biodegradation

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Part of the presented results have been included in the following publication:

- Teixeira PD, Silva VS and Tenreiro R (2019) Integrated selection and identification of bacteria from polluted sites for biodegradation of lipids. International Microbiology. Doi: 10.1007/s10123-019-00109-w

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List of Abbreviations

ANT – Anthracene

AUC – Area Under Curve

ACE – Abundance-based Coverage Estimator

BBC – *BioTask* Bioremediation Collection or bacterial isolate from *BioTask* Bioremediation Collection

BLAST – Basic Local Alignment Search Tool

BOD_5 – Biochemical Oxygen Demand after 5 days incubation at 20ºC

CV – Coefficient of Variation

COD – Chemical Oxygen Demand

DO – Dissolved Oxygen

EPA – United States of America Environmental Protection Agency

FOG – Fat, Oils and Grease

GMO – Genetically Modified Organisms or Microorganisms

GTS – Glyceryl tristearate

HPLC – High Performance Liquid Chromatography

HRT – Hydraulic Retention Time

LLE – Liquid-Liquid Extraction

LOD – Limit Of Detection

LOQ – Limit Of Quantification

MiDAS – Microbial Database for Activated Sludge

MiO – Mixed lubricant oil

MPN – Most Probable Number

NAUC – Net Area Under Curve

NGS – Next Generation Sequencing

OA – Oleic Acid

OD – Optical Density

OUR – Oxygen Uptake Rate

OTU – Operational Taxonomic Unit

PAH – Polycyclic Aromatic Hydrocarbon

PCA – Principal Component Analysis

PCR – Polymerase Chain Reaction

PE – Population equivalent or unit per capita loading for wastewater treatment assessment

PHE – Phenanthrene

RHD – Ring Hydroxylating Dioxygenase

RDP – Ribosomal Database Project

TO – Triolein

TSS – Total suspended solids

WHO – World Health Organization

WWTP – Wastewater Treatment Plant

YBC – Yeast isolate from *BioTask* Bioremediation Collection

Chapter I - General Introduction

1.1 State of the art: wastewater biological treatment and bioremediation

Water treatment is an essential part of human development, for a sustainable and healthy environment. In fact, the wastewater sector contributes significantly to the social and economic development of every country, both by its ability to generate economic activity, create jobs and wealth, and also ensures human health protection. In Portugal, the services in this sector are recognized as essential public services under national law, which are defined by the government (*Lei n. º 23/96, de 26 de Julho*). In 2010, the General Assembly of the United Nations declared access to clean water and sanitation an essential human right to the full enjoyment of life and all other human rights. This recognition implies an obligation on states to respect, protect and secure this right. Reinforcing such idea, the new resolution from the General Assembly of the United Nations in December 2015 recognized basic sanitation as a separate human right from the right to clean water (Resolution A/RES/64/292). In this new resolution, the human right to sanitation recognizes, without discrimination, the right to have physical and accessible access to sanitation, in all spheres of life, that is safe, hygienic, socially and culturally acceptable, and that provides privacy and guarantee dignity. Implementing these rights means that everyone must have adequate and safe access to safe drinking water and sanitation. For these reasons, each national law requires local treatment systems that can effectively treat all the received wastewater, ensuring it does not harm the environment and/or causes long term effects over human health. A healthy and unpolluted environment is a fundamental requirement and a key right for our society growth.

1.1.1 Wastewater Treatment Plants (WWTP)

The engineered solution, applied worldwide for wastewater treatment, are Wastewater Treatment Plants (WWTPs), a designed infrastructure to remove pollutants and reduce their impact on the environment to an acceptable limit. The European Commission (EC) has defined the level of pollution according to several factors (sensibility of the receiving water body, location, water end-use). From these regulations, each country has passed its own national legislation to impose limits and control contamination levels. In Portugal, the specific concentration levels for BOD_5, COD and TSS are set since 1997, for general municipal wastewater (Table 1.1). Since then, more legislation has been approved for industrial and agricultural activities to enforce specific wastewater treatment levels, especially for nutrients and metalloids contamination. WWTP discharge into environment has different legislation requirements according to human health risk and environmental impact on water bodies (rivers, lagoons or sea).

Table 1.1 – Requirements for the discharge of effluent from WWTP under Portuguese law (*Decreto-Lei 52/97*). PE, population equivalent. The listed parameters: biochemical oxygen demand for 5 days (BOD_5), the chemical oxygen demand (COD) and the total suspended solids (TSS).

Parameter	*Concentration limit*	*Minimal percentual reduction*
BOD_5	25 mg/L O_2	70 – 90
COD	125 mg/L O_2	75
TSS	35 mg/L (for PE over 10 000)	90 (for PE over 10,000)
	60 mg/L (for PE between 2,000 and 10,000)	70 (for PE between 2,000 and 10,000)

National legislation is more restrictive for rivers and lagoons, given it serves as a potable water source for human and agricultural use. In Portugal alone there are 2 751 WWTPs, processing over 491 million cubic meters of wastewater annually, according to the official government entity (ERSAR, 2018). Depending on effluent contamination and final end-use for treated wastewater, a WWTP may have up to 7 treatment levels: preliminary, primary, advanced primary, secondary, secondary with nutrient removal, tertiary and advanced (Table 1.2). Each treatment level targets different components: the preliminary and primary focus on the removal of solid residues and sediments, most particulate and inert material; the secondary treatment, also referred as biological treatment, for the removal of organic pollution (dissolved organic matter and/or nutrients – nitrogen and phosphorus compounds) by promoting microbial metabolism; the tertiary and advanced treatments are usually applied to allow specific wastewater reuse, in irrigation for example. During a WWTP regular operation, various sub-products in all phases (liquid, solid and gaseous) must be dealt with (Figure 1.1). Management of all sub-products to appropriate recovery and valorisation, when applicable, or to landfill deposit for complete elimination, is a requirement for sustainable ongoing treatment.

As illustrated in Figure 1.1, the sludges from the primary and secondary treatment can follow a sludge treatment in order to allow valorisation as fertilizer or for potential production of biogas. However, not all WWTP can be directed to such route. For fertilizer production, sludge must comply with strict contamination levels of accumulated pollutants, such as metals (cadmium, lead, copper, chromium, mercury, nickel, zinc) (*Decreto-Lei 103/2015*). For biogas production, only sludge with high carbon content should be applied in anaerobic digestion by methanogenic archaea. In fact, methanogens obtain their energy for growth from the conversion of a limited number of substrates to methane gas (CH_4), where major known substrates are $H_2 + CO_2$, formate, and acetate (Whitman *et al*., 2006).

Table 1.2 – Levels of wastewater treatment according to Tchobanoglous and colleagues (2014)

Treatment level	*Description*
Preliminary	Removal of wastewater constituents such as rags, sticks, floatables, grit, and grease that may cause maintenance or operational problems with the treatment operations, processes, and ancillary systems.
Primary	Removal of a portion of the suspended solids and organic matter from the wastewater.
Advanced primary	Enhanced removal of suspended solids and organic matter from the wastewater. Typically accomplished by chemical addition or filtration.
Secondary	Removal of biodegradable organic matter (in solution or suspension) and suspended solids. Disinfection is also typically included in the definition of conventional secondary treatment.
Secondary with nutrient removal	Removal of biodegradable organics, suspended solids, and nutrients (nitrogen, phosphorus, or both nitrogen and phosphorus).
Tertiary	Removal of residual suspended solids (after secondary treatment), usually by granular medium filters, cloth filters, or microscreens. Disinfection is also typically a part of tertiary treatment. Nutrient removal is often included in this definition.
Advanced	Removal of dissolved and suspended materials remaining after biological treatment when required for various water reuse applications.

However, during the anaerobic production, other components are produced and must be removed before energetic valorisation, including hydrogen sulphide, halogen compounds (chlorides, fluorides), ammonia, siloxanes and volatile organic compounds (VOC's). Such impurities may cause corrosion, deposits and damages to equipment and infrastructure, reducing biogas value. Methane is an important greenhouse gas with a global potential impact greater than CO_2, and for this reason anaerobic digesters must burn the generated methane to reduce environmental impact, even if the energetic valorisation is not economically viable. (Howarth *et al*., 2011; Jiang *et al*., 2018).

1.1.2 Biological treatment in WWTP

The biological treatment implemented in WWTP was based on the empirical data collected from the contamination of natural water streams, which, over time, tend to recover to its original state. This self-purification of water is caused by natural biodegradation of common pollution (organic matter and similar compounds), conducted by bacteria, protozoa, metaprotozoa, and more complex organisms. As previously described, the wastewater engineering solutions focus on infrastructure which improves microbial activity (biodegradation), and/or specific pollutants metabolization and elimination.

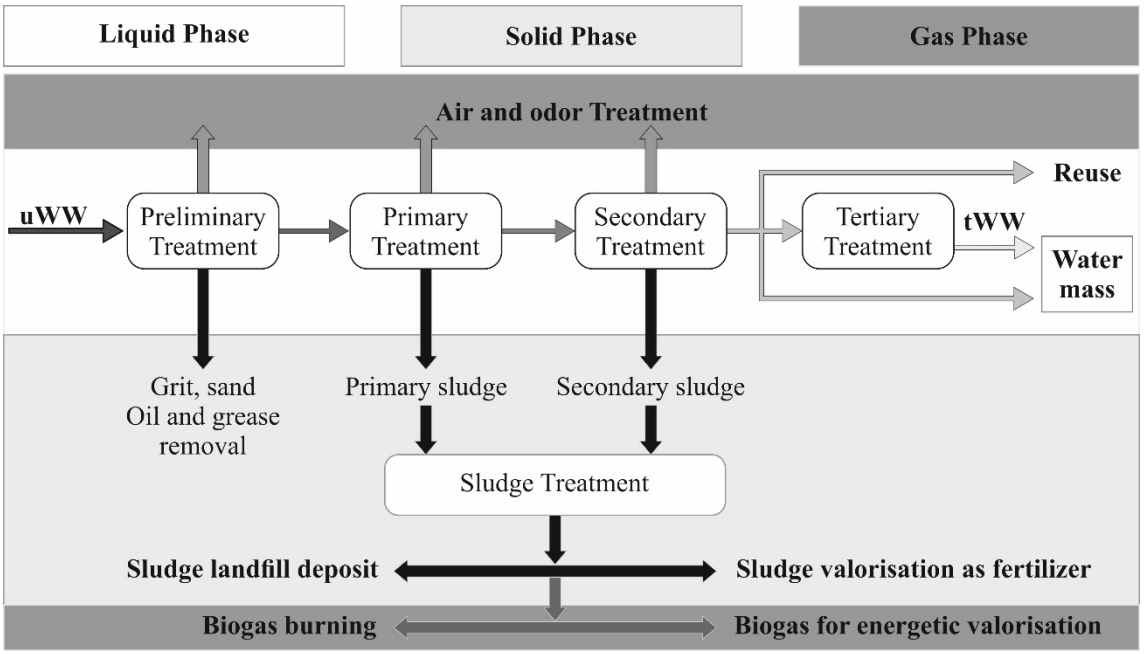


Figure 1.1 – Treatment flow for an average WWTP, with separate lines for liquid, solid and gas phases. (uWW: untreated wastewater; tWW: treated wastewater)

In fact, over the last century several different technologies have been implemented to increase wastewater depuration efficiency in the secondary (biological) treatment process. The most common strategy for WWTP is activated sludge process, where a specialised microbiome obtain from the wastewater is maintained and fed with untreated wastewater in an aeration tank, which promotes aerobic metabolisms and the contact with the wastewater pollutants. Also, the mechanical circulation of biomass (obtained from the solid phase separation of the secondary settling tank) allows to maintain a high level of biomass inside the aeration tank and increases the contact time of the biomass with the wastewater. The microbiological structure of wastewater has been studied in the last decades, to identify and understand the most relevant microorganisms growing in such specific environments. Cultivation methods by direct isolation or serial dilutions plated in general growth media or selective medium were among the first strategies used for microbiological studies in wastewater (Jannasch and Jones, 1959; Prakasam and Dondero, 1967; Banks and Walker, 1977; Dudley *et al*., 1980). However, the direct isolation approach was not fitted to analyse all the WWTP diversity, given the presence of slow and fast-growing microorganisms on solid media, the presence of most microbial groups (virus, archaea, bacteria, fungus and protozoa) and mostly the complex symbiotic relationships created in secondary wastewater flocs as showed in Figure 1.2 (Wagner *et al*., 1993; Sperling, 2007). Additionally, these strategies have shown large discrepancy between total direct microscopy counts and viable plate counts for many ecosystems, as aquatic habitats (Jannasch and Jones, 1959) and specially in wastewater

samples, with recovery numbers as low as 1% (Soddell and Seviour, 1990). More recently, genetic markers (as fluorescence probes and FISH) and other molecular approaches (as 16S rRNA gene sequencing, or NGS) have been applied to study relevant taxonomic groups in wastewater microbiome (Howgrave-Graham and Steyn, 1988; Wu *et al.*, 2019; Brand *et al.*, 2019). These approaches intend to close the gap between the observed microscopic diversity and the low numbers obtained in cultivation methods. The data analyses for wastewater bacteria identification, deepen by the last decade of genomic sequencing of natural samples, using NGS approaches, have shown a quite diverse environment. The results have been explained by the highly diverse natural origins, given that WWTP receive water from several households, industries and, sometimes, multiple agriculture facilities. Some authors (McIlroy *et al*., 2015; McIlroy *et al*., 2017) have successfully identified most prevalent taxonomic groups in wastewater and similar infrastructure. Another difficulty concerns spatial distribution and organization for bacteria, filamentous growth and particles within activated sludge floc, as illustrated in Figure 1.2.

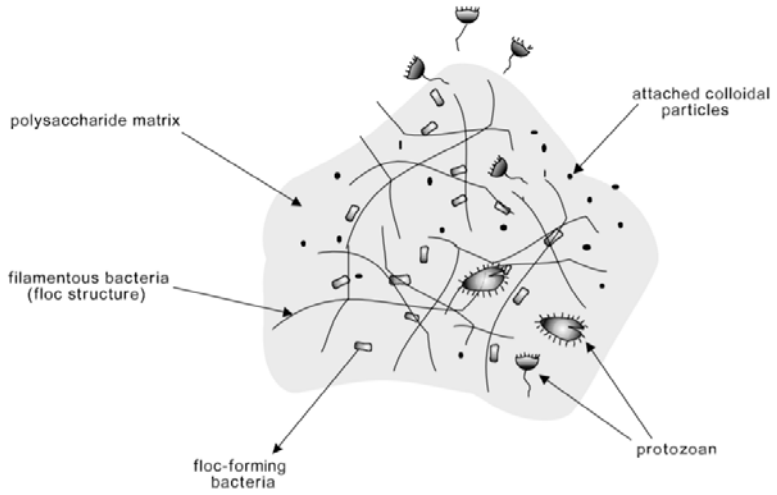


Figure 1.2 – Schematic structure of an activated sludge floc (from Sperling, 2007)

The "floc" organization represents a protective structure for microorganisms, composed by an exopolysaccharides (EPS) matrix “trap” (Sam and Dulekgurgen, 2016), surrounding bacterial cells, filamentous growth and suspended organic matter. These structures allow anaerobic metabolism in the centre and aerobic metabolism in the peripheric area, as well as physical support and connection among different bacteria, fungi and protozoa. Also, it increases the superficial contact area of microorganisms with wastewater pollutants, and reduces predation from higher trophic organisms, such as protozoa, which feed on single-cells organisms (Sperling, 2007). These structures can be very small (around 10 μm) to quite large (≤1 mm),

supporting many different microbial species. In a mature activated sludge from WWTP, higher taxa organisms, as protozoa, are reported biological indicators to evaluate the process activity and health. In fact, the presence of large flocs, populated by ciliated protozoa (both crawling and attached) are associated to high biodegradation levels and nitrifying activity by the biomass (Madoni, 2011). The biological treatment has been widely applied for its simplicity, low investment in infrastructures and maintenance cost over more specific, highly efficient and high cost chemical approaches which usually leads to toxic end-products, as chemical precipitation. Using microorganisms to treat wastewater pollution presents the easiest and cost-effective strategy to deal with nonspecific pollution, by effective degradation and metabolization of pollutants, incorporating then in the natural cycles. On the other hand, physical and chemical approaches for wastewater purification, by compounds precipitation and filtration, leads to toxic by-products, usually ending in landfill deposit or incineration. Though no universal technology can solve all man-made pollution, today strategy usually relays on biological activity for pollution elimination.

1.1.3 Bioremediation

Considering the most recent definition for bioremediation (a process that uses living organisms, microorganisms and/or plants, to degrade, reduce or detoxify waste products and pollutants, in Gouma *et al*., 2014), the conventional WWTP technology should be considered as a bioremediation plant. However, over the last decade, authors tend to refer to bioremediation only for contaminated soils treatment, or specific pollutants removal, as crude oil spills, heavy metal or polychlorinated biphenyls decontamination (Dott *et al*., 1995; National Research Council, 1993). A schematic representation of bioremediation is presented in Figure 1.3, considering its broadest definition, and referring to all approaches involving biological activity towards detoxifying or decontamination of a specific site, water body or air stream. It can be divided according to the effective location were the biodegradation activity takes place, whether *in situ*, near the production site for the treated xenobiotic or the polluted site; or *ex situ*, in specifically constructed infrastructure to retain the toxic waste or polluted water, where is subjected to the biological activity. The simplest example of *in situ* bioremediation is natural attenuation of a contaminated site, which results by monitoring the biodegradation activity of the naturally occurring microorganisms over time.

Several other strategies have been followed to promote microbial decontamination, from biopiles to landfarming (*ex situ*) or biostimulation to phytoremediation (*in situ*), favouring different conditions for biological activity and presenting multiple engineering challenges.

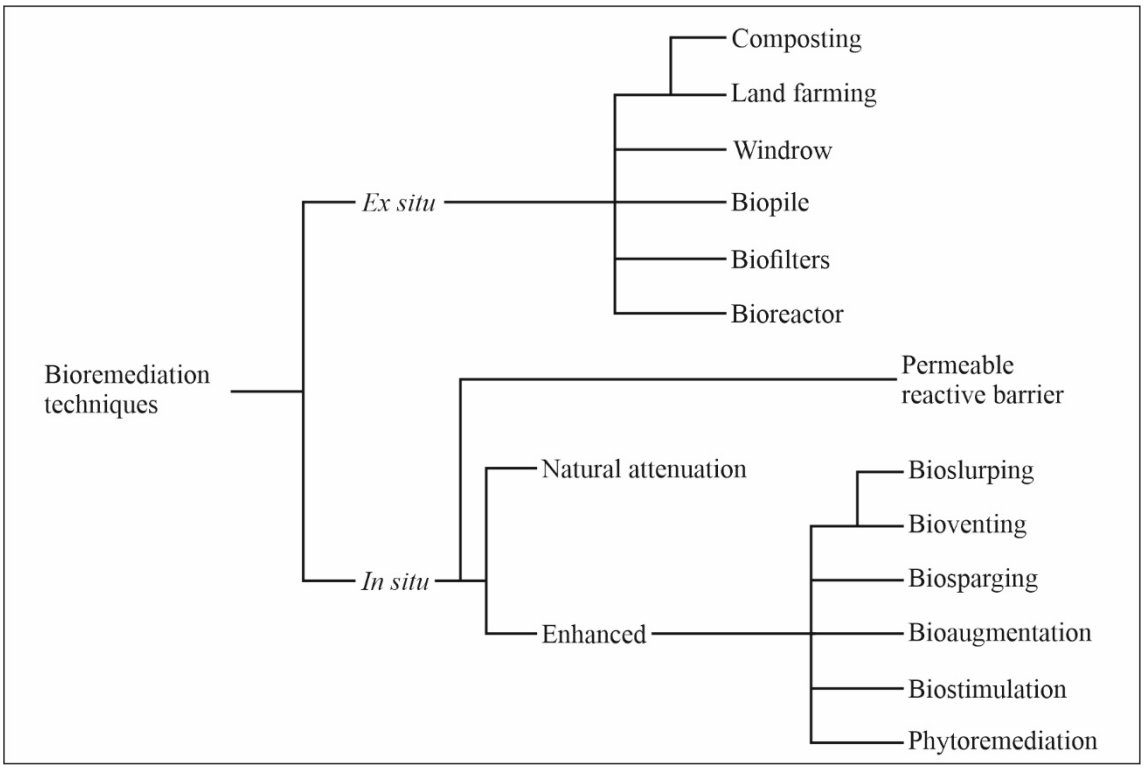


Figure 1.3 – Bioremediation approaches (adapted from Boopathy, 2000 and Azubuike *et al*., 2016)

For example, the permeable reactive barrier is a remediation technique where a physical barrier is implemented to retain contamination, presenting some elements of *in situ* bioremediation but not fully directed. The enhanced approaches present the most engineered methodologies, actively promoting pollutants elimination by biological activity (mostly bacteria). For bioslurping, bioventing and biosparging, forced air introduction into different levels promotes pollutants removal and/or degradation, whether by physical removal or by microbial activity. Biostimulation corresponds to chemical correction (pH, limiting nutrient, water content, etc) to improve natural microbiome biodegradation of hazardous compounds. Bioaugmentation is based on the introduction of exogenous microbial cultures (bacteria and/or fungi), containing the necessary metabolic routes to complete pollutants removal. There is a growing worldwide market for biotechnological based products with microorganisms, mostly bacteria (*Bacillus* spp. and *Pseudomonas* spp., according to manufacturers) to promote biodegradation in WWTP (Brooksbank *et al*., 2007; Tzirita 2012). The phytoremediation strategies are based on plants to remove pollutants from site, whether by direct biomass incorporation, as some authors have shown for heavy metals, or by promoting biological barrier in rhizosphere and promoting specific microbial population growth in such harsh environment (DalCorso *et al*., 2019). To avoid confusion over the definition of approaches, a summary is presented in Table 1.3.

Table 1.3 – Different bioremediation approaches targeting different treatment phases: liquid, solid and or gaseous.

Approach	*Phase*	*Description*	*Reference*
Composting	Solid	Involves mixing contaminated soil with manure, food wastes or agricultural wastes. Biodegradation occurs by microbial population growth, with temperature increase, characteristic of a composting process.	Amir *et al*., 2005; Kumar *et al*., 2011; Mani and Kumar, 2014
Land-farming	Solid	Contaminated site is excavated up to 30 cm, to stimulate the aerobic activity of indigenous microorganisms. The monitoring and maintenance costs are reduced, as well as the treatment liabilities.	Lynch and Moffat, 2005; Kumar *et al*., 2011; Mani and Kumar, 2014
Biopile	Solid	A combination of landfarming and composting. The contaminated soil is mixed with agricultural wastes or similar and maintained in piles. Stimulates aerobic and anaerobic microbial degradation, and allows surface treatment, leaching control and substrate volatilization.	Lynch and Moffat, 2005; Kumar *et al*., 2011; Mani and Kumar, 2014
Biofiltration for waste gas treatment	Gas	Specifically design for biological treatment of volatile pollutants. A filter material (compost, peat, bark, or inert material) gives support to microorganisms to contact with waste gases passing through. The reactor allows target organic pollutants to diffuse into the biofilm promoting aerobic biodegradation.	Tang *et al*., 1996
Bioreactor	Solid or liquid	An engineered containment system with controlled conditions to favour aerobic metabolism by natural microbiome. Can incorporate soil, sediments, sludge or water where the biodegradation-controlled conditions of the contaminants are established in an aqueous suspension.	Lloyd and Lovley, 2001; Santos and Judd, 2010; Kumar *et al*., 2011; Mani and Kumar, 2014
Permeable reactive barrier	Liquid	By forcing contaminated water to pass through a reactive barrier constructed to retain the contaminants. The most common reactive materials are carbon-based materials as activated carbon and plant-derived biomass, zeolite or, more recently, graphene. Reactive permeable barriers will physically support microorganisms, promoting natural degradation.	Thiruvenkatachari *et al*., 2008
Natural attenuation	Solid and liquid	Taking advantage of indigenous microbial populations and with the minimum impact, focus on monitoring pollution biodegradation over time.	Kumar *et al*., 2011; Mani and Kumar, 2014
Bioslurping	Liquid and gas	Also referred as multi-phase extraction. Used to remove the free-floating product on a water table by combining vacuum-enhanced free product recovery with bioventing. It is mainly designed to control the environmental release of soil gas and groundwater.	Senthil and Gunasundari, 2018
Bioventing	Solid and liquid	Air is slowly pumped into contaminated soil through injection wells, to promote aerobic biodegradation by natural microbiome. Used if geological conditions favour air dissipation.	Lynch and Moffat, 2005; Kumar *et al*., 2011; Mani and Kumar, 2014
Biosparging	Liquid	By forcing air injection below the water table (saturated zone) to raise the oxygen concentration in groundwater, biodegradation rate is increased by naturally occurring bacteria.	Senthil and Gunasundari, 2018
Bio-augmentation	Solid and liquid	Technology that involves the addition of exogenous microbial cultures into the contaminated site. It is frequently used to accelerate bioremediation in conjugation with other approaches.	Kumar *et al*., 2011; Mani and Kumar, 2014; Dixit *et al*., 2015
Bio-stimulation	Solid and liquid	Involves the addition of rate-limiting C/N sources or supplements to stimulate the growth of indigenous microbial population, capable of degrading the contaminants from soil and/or aquatic environment.	Mani and Kumar, 2014; Dixit *et al*., 2015
Phyto-remediation	Solid and liquid	By exploiting plants capacity to remove contaminants from polluted environments. It is eco-friendly, dropping soil erosion, improving soil fertility by increasing organic matters in soil.	Bharathiraja *et al*., 2018
Pump and treat	Liquid	Groundwater can be pumped, treated and returned after pollution reduction. It represents the most expensive investment and maintenance, and high time-consuming process.	Senthil and Gunasundari, 2018

The selected approaches present the most commonly referred strategies to promote biological biodegradation, although other authors may present slightly different definitions. Overall, the main differentiating features for bioremediation processes are: where they take place (*in situ*

or *ex situ*), how the biological activity is promoted (passively or actively), and, when actively, which engineering solution is applied (forced air, irrigation, heating, and/or mixing, etc).

Considering the accepted definition for bioremediation, and considering the conventional WWTP, one must include the biological treatment techniques (as activated sludge treatment, percolate filters, biological filters, anaerobic digestion, and others) as bioremediation approaches. They are all based on microbiological activity to remove wastewater pollution, promoting the presence of specific microbial population to promote aerobic or anaerobic degradation, engineering infrastructure to increase HRT, creating a stable and homogenous effluent, and/or specific physical support for microbial populations maintenance (Henze and Comeau, 2008). However, WWTP are not commonly associated to the concept of bioremediation, as they are recognized as part of sanitary engineering, where the main concern relays on physical and mechanical optimizations (HRT, biomass circulation, suspended solids, aeration, oxygen level, etc) to increase pollution removal efficiency. An important interconnection between both areas should be reached, since one of the recognized problems for unconventional bioremediation strategies implementation in pilot-scale are its efficiency determination and regulatory approval. As stated in Table 1.4, there are overall advantages in applying multiple bioremediation strategies, to achieve the best possible result.

Physical and chemical treatment processes for pollutants removal create other problems, since filtration, chemical precipitation or transformation, lead to toxic end-product, increasing the chance of future liability with highly contaminated and concentrated residues (Crini and Lichtfouse, 2019). Bioremediation techniques are more economical than traditional physical and chemical methods and can achieve complete degradation of organic pollutants without destructions of the site material or its flora and fauna. Also, being economical, can be used *in situ* for removal of pollutants at low but environmentally significant concentrations, preventing gradual build-up. Furthermore, pollutants can be treated *in situ*, thus reducing exposure risks for clean-up personnel or potentially wider exposure as a result of transportation accident (Hawumba *et al*., 2010).

Bioremediation has proven to be a valuable and cost-effective solution (Madigan *et al*., 2010). By introducing new microbial consortia in contaminated environments (bioaugmentation), the remediation of a contaminated site or wastewater has been effectively achieved (Das and Chandran, 2011; Bhuvaneswar *et al*., 2012). There are commercial products based on catabolically relevant microorganisms, available for household and industry applications (Brooksbank *et al*., 2007). However, uncertain results in field tests have delayed general use of these products.

Table 1.4 – Advantages and disadvantages of bioremediation (adapted from Kumari *et al*., 2018a)

Advantages
Bioremediation is a natural process and tends to have wide public acceptance for toxic waste treatment.
Overall, bioremediation depends on low infrastructure, has lower cost and requires less energy.
The final residues from microbial degradation of hazardous wastes are harmless products such as carbon dioxide, water and cell biomass.
Microbial degradation allows complete destruction of a wide variety of toxic hazardous pollutants.
Degradative populations increase during the biodegradation process and tend to decline after contaminant removal.
Bioremediation allows to convert legally hazardous pollutants into harmless products, eliminating the chance of future liability with contaminated material.
Microbial genetic approaches have allowed to develop highly efficient microbes for degradation of specific hazardous pollutants, to fulfill incomplete natural degradation or improve species efficiency and/or tolerance levels.
Bioremediation causes less disruption of natural environment impact, since microbial degradation is an environmentally friendly approach.
Disadvantages
Not all the toxic hazardous waste is susceptible to rapid and complete microbial degradation.
More toxic and persistent intermediate metabolites may be formed during microbial degradation.
Microbial degradation requires precise conditions such as suitable environmental settings (pH, temperature, water content, etc.), capable microbial populations and appropriate levels of nutrients and contaminants.
Difficult to infer the efficiency of microbial degradation from laboratory scale to pilot scale.
Quite slow process, might require months to years to clean-up the environment, depending on target and contamination levels. Requires more time than other physicochemical technologies for degradation of toxic wastes.
Regulatory imprecision over performance for bioremediation. Also, efficiency evaluation is difficult, specifically at pilot-scale, and there are no acceptable endpoints for bioremediation treatments.
A stronger scientific base is required for rational designing of process and success for genetic engineered microbes (GMOs)
For the successful *in situ* degradation of toxic hazardous waste, the contaminated site must contain soil with high permeability to reduce contamination leaking.

Field-tests are influenced by wastewater variability in chemical composition, presence of inhibitors, pH, temperature and the naturally developed community. To achieve bioremediation of wastewater through bioaugmentation, the introduced microorganisms must present highly efficient catabolic relevant features and also persist as a stable population in a highly competitive and dynamic environment as wastewater systems (Thompson *et al*., 2005). Commercial products for bioaugmentation provide viable microorganisms, mostly isolated from highly contaminated sites, subject to acclimatization before isolation or simply

presenting relevant catabolic features, to complete contaminants biodegradation (Shon *et al*., 2002; Brooksbank *et al*., 2007; Zanaroli *et al*., 2010).

Today engineering strategies focus on accommodate solid state pollution in landfills, which are prone to soil erosion and leachate diffusion to soil and groundwater contamination (Lee *et al*., 2019). On the other hand, wastes incineration has been associated to increase in air pollution, soil contamination and human health hazard for affected areas (Ma *et al*., 2018).

As previously described, commercial products are available for bioremediation purposes, based on viable enzyme producing microorganisms. In order to positively impact wastewater treatment, these microorganisms must re-establish microbiome metabolic capacity, reorient the microbial dynamics towards pollution remediation and influence biomass sedimentation, to promote pollution removal in an efficient way. A fundamental step for new bioremediation product design is strain selection to respond to specific pollutants. In fact, wastewater microbial diversity is still a growing field study, with increasing information, day-by-day, from genomic data. However, a selected strain must create a viable, long-term population, in a highly dynamic, competitive and restrictive environment such as wastewater, while promoting pollutant metabolization, without negative effect over naturally existing microbiome.

1.1.4 Metabolic pathways for pollutants

Water soluble substrates as pentoses (arabinose, ribose, xylose), hexoses (glucose, fructose, galactose) and other saccharides (saccharose, lactose, maltose, trehalose) are easily assimilated by microorganisms, when presenting the necessary metabolic pathway. This can be explained by the easy access and transport mechanism for such diverse molecules. Similarly, most soluble organic matter is available for microbial biodegradation and removal in WWTP. However, insoluble or highly hydrophobic substrates present an added challenge for microorganisms. Most common hydrophobic compounds in wastewater are triacylglycerols, a waste from food and feed industries, and also household kitchens; and hydrocarbon residues (composed only by carbon and hydrogen, being linear molecules as *n*-alkanes, or cyclic as polycyclic aromatic hydrocarbons, PAH), mostly from petrochemical, automobile, and transformation industries, with high impact on environment and human health. Triacylglycerols, also referred as FOG (fat, oil and grease), are associated with sewage pipes clogging as well as reducing oxygen transfer in WWTP biological components, disrupting continuous wastewater treatment process (Brooksbank *et al*., 2007). Hydrocarbons are of intense public concern owing to their environmental persistence and potentially deleterious effects on human health (Facchin *et al*., 2013; Watanabe, 2001). For such reasons,

several microorganisms (both bacteria and fungi) have been studied for possible biodegradation pathways, to determine the essential enzymes involved.

For PAH aerobic biodegradation, critical attack occurs by initial oxygenase action, as shown in Figure 1.4. The degradation pathway starts with oxygenase enzyme (mono- or di-), which enables the aromatic ring disruption and allows the subsequent reactions. If the molecule contains multiple rings, sequential oxygenases act on each molecule to produce linear hydrocarbon or phenol and/or catechol residues. These simpler molecules can then be incorporated in specific metabolic pathways, as exemplified in Figure 1.5. Some fungi and bacteria contain a cytochrome-P450 monooxygenase-parallel, while PAH dioxygenases have only been described in bacteria (Cerniglia, 1992; Haritash and Kaushik, 2009). The proposed pathways in Figure 1.4 can be found in bacteria and/or fungi, providing evidence for biological degradation of such substrates.

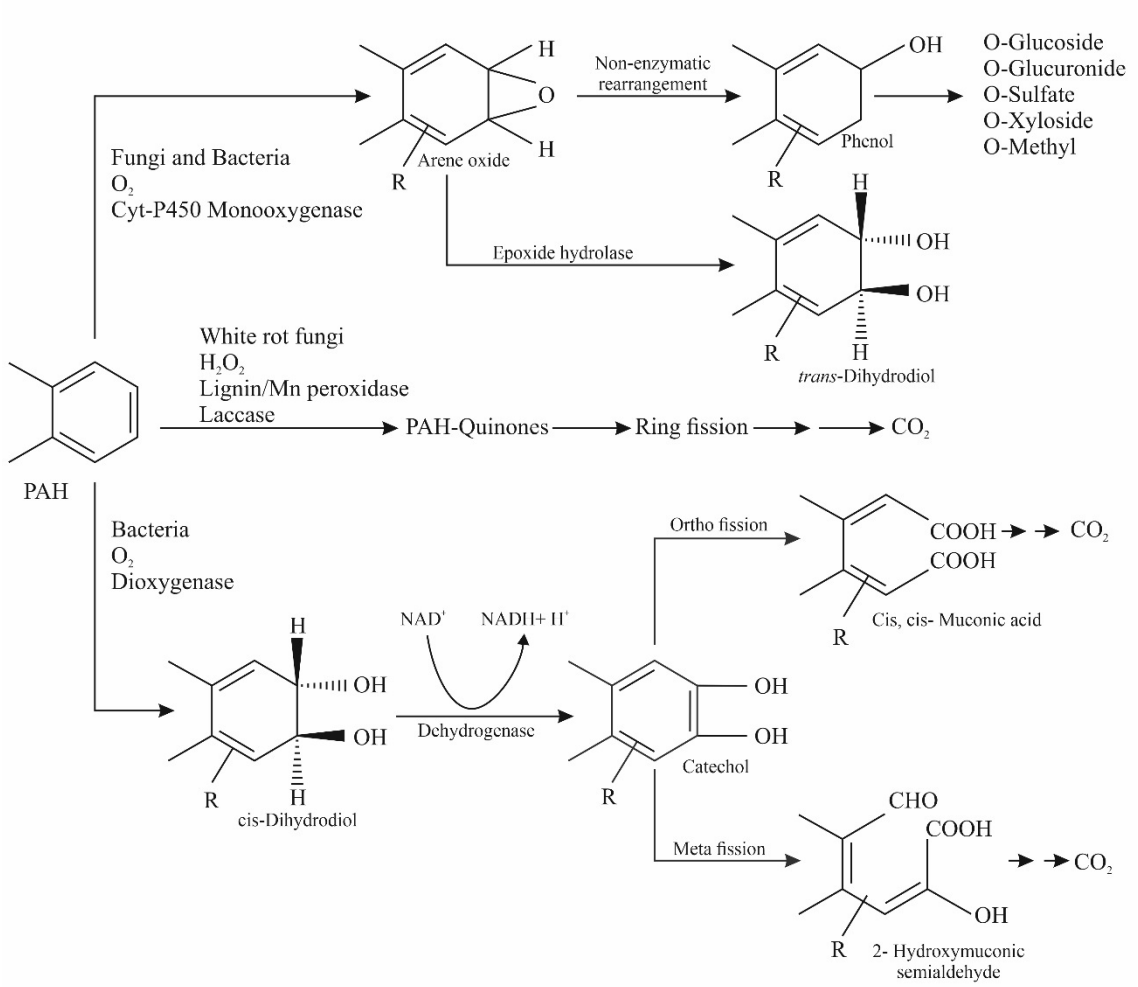


Figure 1.4 – Proposed pathways for aerobic microbial catabolism of polycyclic aromatic hydrocarbons (adapted from Haritash and Kaushik, 2009; Cerniglia, 1992)

However, theses substrates are considered recalcitrant in nature, given the low natural biodegradation by resident microbiome in WWTP or contaminated soil. This results from the low solubility of these compounds, which reduces its availability for enzymatic activity. The first oxygenase step limits downstream metabolic pathway for degradation of hydrocarbons. Given that enzymatic activity works in water dissolved substrate, it only affects a small part of the contaminant, requiring it to slowly be transported inside the cells after conversion into smaller molecules.

For FOG contamination, main limiting step are lipase/esterase activity, which convert insoluble triacylglycerols into fatty acids. Only then can the fatty acids be available for further intracellular enzymatic transformation. The previous metabolic pathway was described in oleaginous yeasts, as *Yarrowia lipolytica* (Figure 1.5), capable to degrade, produce and accumulate several substrates in lipid bodies. Initial enzymatic routes for extracellular oxygenase, lipase or esterase activity, prior to residues incorporation was also described for bacteria (Jaeger *et al*., 1994; Wentzel *et al*., 2007).

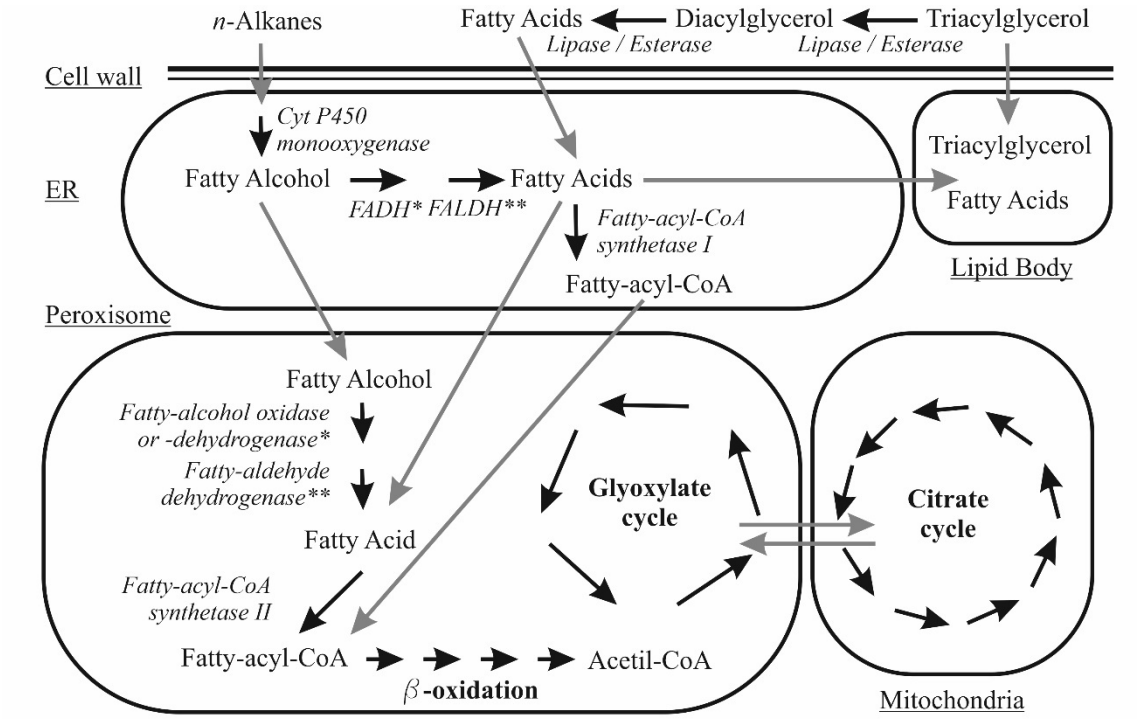


Figure 1.5 – Main metabolic pathways and cellular compartments involved in hydrophobic-substrate degradation in *Yarrowia lipolytica*. Hydrophobic substrates (*n*-alkanes, fatty acids, triacylglycerols) enter the cells via unknown uptake systems (grey arrows indicate transport processes). Alkanes are first oxidised by cytochrome P450-dependent alkane monooxygenase systems in endoplasmic reticulum (ER) and further converted by fatty-alcohol-oxidase and dehydrogenases, in two steps into corresponding fatty acids in peroxisomes or in ER (black arrows indicate enzymatic activity). Triacylglycerols can be actively accumulated in lipid bodies or extracellular conversion into fatty acids by lipase or esterase action. Fatty acids are substrate for either fatty-acyl-CoA synthetase I in ER or peroxisomal fatty-acyl-CoA synthetase II prior to entry into the *β*-oxidation pathway. Formed acetyl-CoA enters the glyoxylate-cycle pathway in peroxisomes, which interacts with the citrate cycle, located in mitochondria. Enzymes are in italics and cellular compartment are underlined (adapted from Fickers *et al*., 2005)

1.1.5 Adaptive evolution experiments

Experimental adaptive evolution is a method used to promote changes, by basic molecular evolution and/or adaptive changes, accumulated in microbial populations over long timeframe, when maintained under defying growth conditions. In the present work, adaptive evolution experiments (AEx) of a microbial community were directed for the enhancement and screening of insoluble substrate degraders. To my knowledge, this was the first time this improvement strategy was applied to a natural microbial consortium for the isolation of enhanced strains for bioremediation purposes. As stated, adaptive evolution is based on cumulative and gradual small changes in a specific strain or microbial community, when maintained in a stress-inducing condition. The improvement has been associated to small genetics variants (SNP's or indels), or even large genomic alterations (gene or plasmid duplication or deletion), as illustrated in Figure 1.6.

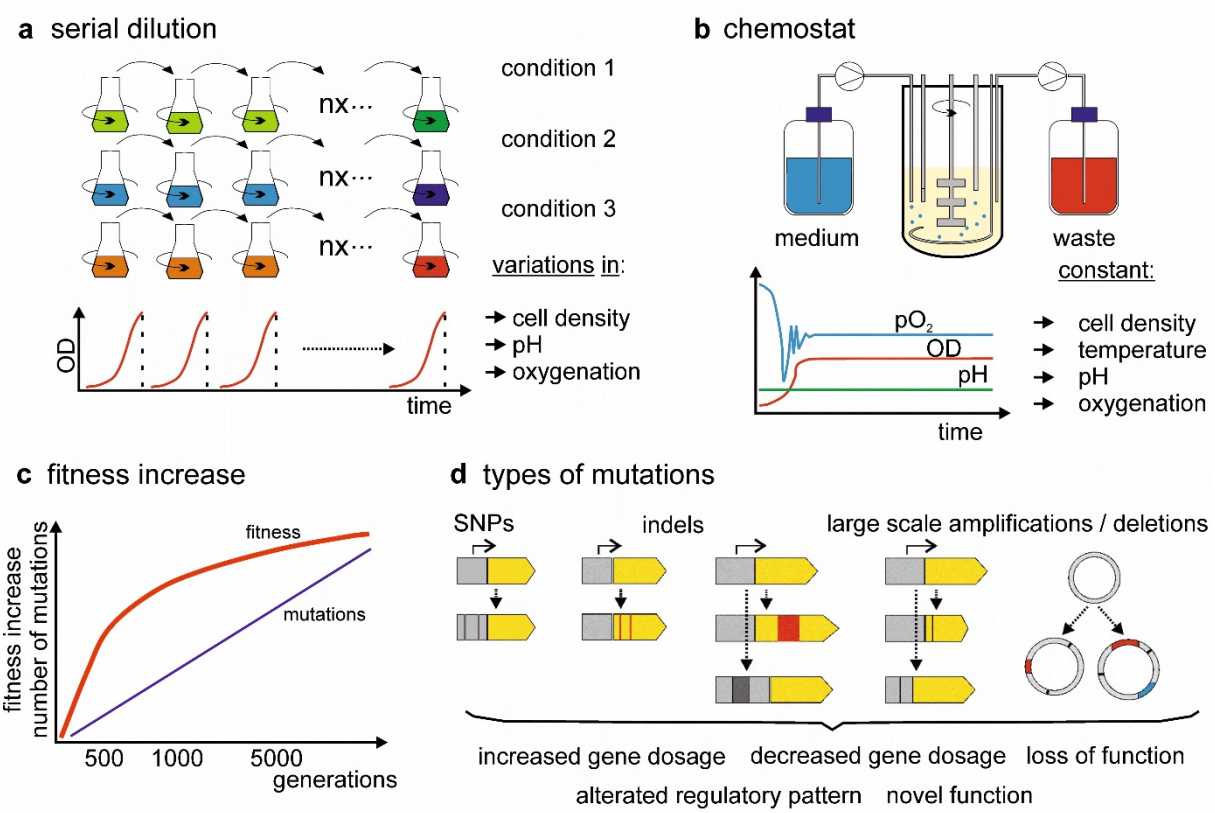


Figure 1.6 – Adaptive evolution experiments. AEx can be performed in the laboratory: (**a**) sequential serial passages in shake flasks; (**b**) chemostat cultures, where one nutritional component is typically limited, and cell density can be much higher than in shake flasks. Additionally, cell density and environmental conditions can be kept constant and more complex cultivation strategies can be implemented; (**c**) the increase of fitness during evolution experiments is fast in the first stage but generally slows down during prolonged selection, whereas the number of mutations is steadily increasing; however network complexity leads to a decreasing beneficial effect of additional mutations; (**d**) mutations that are usually identified in AEx studies: Single Nucleotide Polymorphisms (SNPs), smaller insertions and deletions (indels) and larger deletions and insertions contribute to genetic and gene regulatory changes and fitness changes during the selection for improved phenotypes (adapted from Dragosits and Mattanovich, 2013).

Previous authors have successfully applied such strategy for higher tolerance to ethanol, acetic acid, temperature (Wallace-Salinas *et al*., 2013; Horinouchi *et al*., 2015; Shui *et al*., 2015; Li *et al*., 2019), and even higher productivity and growth rates (Fong *et al*., 2005; Mo *et al*., 2019). The approach can be applied in serial dilutions, maintained in flasks or in a chemostat, for the same purpose: keeping an initial inoculum in the tested conditions to force fitness changes (Dragosits and Mattanovich, 2013). In this work, a natural consortium was maintained in a mineral media containing a pollutant as sole carbon source, serving as the driving evolution force towards degraders enhancement. By successively re-inoculating the consortium in fresh medium for numerous cycles, the population tend to stabilize, forcing the survival of more adapted, resistant and efficient strains to biodegrade the pollutants (Turcotte *et al*., 2012). A periodic isolation effort generated a microbial collection, recovered from re-inoculation of 110 cycles, using selective solid growth media to isolate degraders.

To gain insights into the population shifts over time, the latest Next Generation Sequencing (NGS) approach has been applied to follow both functional genes and taxonomic diversity changes. Such fine-tune methodology allowed to study diversity evolution along the AEx, and the stress-inducing effect over different taxa (Stapley *et al*., 2010; Brockhurst *et al*., 2011; Horinouchi *et al*., 2015; Li *et al*., 2019).

1.2 Objectives for this work

Conventional WWTP worldwide depend on biological activity, by maintaining a specialized microbiome to efficiently remove pollutants from wastewater. Today, companies worldwide supply microbial based products for wastewater bioremediation process, known as bioaugmentation. However, such products have shown low diversity and fitness towards specific pollutants removal, such as FOG and PAH. The aim of the present work focused on the development of new microbial inocula for bioaugmentation strategies, using alternative isolation experiments as source for superior strains from naturally occurring microbiome. Bearing in mind the main objective, four specific tasks were considered to reach this goal, namely:

1. Selection of natural consortia, from highly contaminated environmental samples, capable of degrading FOG pollutants. The selected natural consortia presented high potential as a source of microorganisms towards bioremediation strategies.

2. Obtain improved microbial strains through adaptive evolution experiments, by exposing the selected natural consortia to a stress-inducing environment over long periods of

time with pollutants as sole carbon source. A collection of more adapted, resistant and efficient microorganisms was evaluated for its potential application in wastewater treatment.

3. Design new microbial consortia for new bioaugmentation product, aiming for efficient biodegradation of PAH rich wastewater and FOG contaminated effluents.

4. Compare the new designed consortia with other commercial products, in laboratory conditions and field tests, using wastewater residues from local industries.

The execution of these tasks aims to promote novel and improved products with increased added value for environmental biotechnology. The present work fits in the strategic plan of the company *BioTask, Biotecnologia Lda*, by allowing the connection to Portuguese research laboratories and setting the basic methodologies for the establishment of a Research and Development Department (R&D) inside the enterprise. The developed methods were of utmost importance for product development in new bioaugmentation products design and testing, representing a milestone for future scale-up production of unique high value products, filling a biotechnological niche market in Portugal. These specific tasks aimed to create a culture collection comprised of microbial degraders, with broad metabolic capacity and low nutrient requirements for future pollutants biodegradation products or differentiated biological processes.

1.3 Strategic workflow

In this PhD research project, two main approaches were applied for microbial isolation of degraders: direct isolation from man-made polluted samples (described in Chapter II), and isolation from adaptive evolution experiments to promote biodegraders enhancement (in Chapter III). For the first methodology, several natural microbial samples, collected from different WWTPs, were used as a source for new isolates. These isolates were screened for the ability to grow with proxy compounds for FOG (triolein and oleic acid) as sole carbon source. Contaminated environmental samples were screened to increase the chances to isolate interesting microbial degraders for the tested pollutants. A microplate growth screening was conducted with liquid substrates for the natural isolates, to select by lipolytic activity. To assess microbial diversity and allow genomic differentiation among isolates, a PCR-fingerprinting method was also applied. Chapter II covers the methodology and results obtained by such strategy, with main results analysis and conclusions.

A second and innovative approach, applying adaptive evolution experiments (AEx), were directed for enhancement and screening of degraders of solid and insoluble substrates. A

natural consortium was maintained in a mineral media containing a pollutant as sole carbon source, serving as the driving evolution force towards degraders enhancement, by successively re-inoculating the consortium in fresh medium for numerous cycles. A periodic isolation effort generated a microbial collection, recovered from 110 cycles, using selective solid growth media to isolate degraders. All obtained isolates were briefly characterized at phenotypic level (cell shape, Gram staining, catalase and oxidase test) and analysed by multiple PCR-fingerprinting (using primers csM13, PH and $[GTG]_5$), to infer genomic relationships and diversity and detect indistinguishable isolates. To get a glimpse on consortium diversity dynamics, a 3rd generation NGS approach was also applied, for both bacteria and fungi (targeting 16S and 26S rRNA genes), and obtained data were cross analysed with the results from solid media isolation. Detailed methods and results are extensively discussed in Chapter III.

Selected isolates were identified by sequencing of taxonomically relevant genes (coding for bacterial 16S rRNA or fungal 26S rRNA) and the information predicted from taxa allocation was used to infer any limitations of potential application. For new bioaugmentation consortia or product design, selected strains must show higher efficiency and stability of acquired features, present low biological liability (in biosafety level 1, regarded as non-pathogenic), no genetic modifications (no GMO), high storage shelf-life and allow desiccation tolerance for dried products.

Chapter IV focus on the efficiency comparison among selected isolates, where quantification methods were defined and tested for the selected pollutants. The low solubility and the solid state of these compounds restrict the use of common techniques, requiring the initial total extraction of the samples (*liquid-liquid* or *solid-liquid* extraction) with a nonpolar solvent (as *n*-hexane; methyl-tert-butyl ether; trichlorotrifluoroethane), followed by recovery of solvent and quantification by *High Performance Liquid Chromatography* (HPLC), or infrared partition, or *Gas-Chromatography* (GC), or gravimetrically after solvent evaporation (APHA, 2017). Even though standardised approaches are defined, these methods present high variability, specially between laboratories. For this reason, each laboratory should implement and test the quantification method for stability concerns. These methods are of crucial importance for experimental set-up and design of the biodegradation assays, which rely on complete sample extraction and pollutant analysis for each time point. Different methodologies were defined, tested and applied to evaluate isolates activity and select the best degraders for bioremediation application. From indirect observance, and respirometry assays for indirect biodegradation assays, to chemical extraction of FOG and/or PAH, all methods

were fundamental to achieve the proposed goal: define new microbial strains for innovative bioaugmentation approaches. Further information regarding methodologies and results are thoroughly discussed in Chapter IV.

The final Chapter focus on the innovation relevance and integration of this work for both scientific enrichment and niche market opportunity for biotechnology. The close involvement of the Portuguese company *BioTask* was determinant for the main fundamental research followed, to respond to common environmental challenges. Our industry and food production cause real environmental problems affecting our lifestyle and health. Biotechnology has proved to be a reliable option for biological solutions in other fields, where the environment has been somehow mistreated. In Chapter V a main perspective over these topics and relationships with the national market are discussed.

Chapter II - Integrated selection and identification of bacteria from polluted sites for biodegradation of lipids

2.1 Introduction

Food and feed industries produce oily effluents, with high fat, oil and grease (FOG) content, causing serious problem for infrastructures and biological treatment systems (Ducoste *et al*., 2008). Tube clogging by oily residues deposition and interaction causes serious costs for wastewater management companies, while interferes with oxygen dissolution and reduces microbial activity in biological treatment systems (Shon *et al*., 2002; Wallace *et al*., 2017). Several bacterial species have already been studied for FOG biodegradation capacity, namely: *Acinetobacter lwofii, A. calcoaceticus, Aeromonas punctata*, *Bacillus amyloliquefaciens, B. licheniformis, B. pumilus, B. subtilis*, *Bukholderia* spp., *Nitrosomonas* spp., *Pseudomonas aeruginosa*, *P. pseudoalcaligenes*, *Serratia marcescens*, *Staphylococcus aureus* and *Raoultella planticola* (Mongkolthanaruk and Dharmsthiti, 2002; Cammarota and Freire, 2006; Matsumiya *et al*., 2007; Loperena *et al*., 2009; Prasad and Manjunath, 2011; Sugimori *et al*., 2013; Sarmurzina *et al*., 2013; Emeer *et al*., 2014). The yeast species *Yarrowia lipolytica* is also able to use FOG as the sole carbon source (Fickers and Nicaud, 2013; Barth, 2013).

Bioaugmentation, defined as the addition of exogenous microbial inocula to enhance biodegradation (Boopathy, 2000), has been proposed to reduce FOG contamination and companies worldwide offer designed bacterial inocula to establish bioaugmentation strategies (Tang *et al*., 2012; Tzirita, 2012). These products must not contain microorganisms recognized as pathogens and should present a long shelf-life, preferably without refrigeration requirements. For such reasons, many commercial inocula, single or multi-strain based, contain mostly members of *Bacillus* and closely related genera (Brooksbank *et al*., 2007; Tzirita, 2012).

Wastewater treatment plants (WWTP) possess highly diverse microbiomes, usually with Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes as the most abundant bacterial phyla, associated to effluent characteristics and affected by infrastructure management and treatment process (Hu *et al*., 2012; Voolaid *et al*., 2017). Previous studies reported beneficial overall results after commercial inocula addition in pilot and real scale wastewater applications, particularly when such inocula contain microorganisms isolated from ecological niches analogous to the polluted areas. Although some short-term effects on natural microbiome have been reported, such case-studies could be explained by inappropriate species, reduced hydraulic retention time leading to cellular wash-out, reduced strain fitness, or insufficient initial inocula (El Fantroussi and Agathos 2005).

The work presented in Chapter 2 focuses on the selection of bacteria for FOG bioaugmentation, collected from man-made polluted sites. By comparing these new bacterial

isolates with those obtained from commercial products, potential improvement could be assessed. Through an integrative analysis, the isolates with the highest potential biodegradation ability were selected and applied in biodegradation experiments to evaluate FOG removal efficiency. These selected bacteria demonstrate important features for new bioaugmentation products for FOG contaminated effluents.

2.2 Materials and methods

2.2.1 Sampling

Activated sludge from secondary settlers and wastewater effluent samples were used as natural consortia to isolate microorganisms with high bioremediation potential towards FOG removal. Samples were collected from 9 different wastewater sources located in Portugal. Samples ranged from WWTPs treating petrochemical, municipal, food and fish-canning industry effluents, and also grease and hydrocarbon separator samples from fast-food restaurant and mechanic station, respectively. The selected sources represent sites continuously contaminated by FOG compounds from human activity, being expected to be rich in well adapted FOG degrading microorganisms. All samples were stored in individual bottles at 4°C and screened in the following 48 h to guarantee maximum viability. The same strategy was applied to commercial bioaugmentation inocula for comparison purposes, using a 1 g/L suspension. Prior to use, each sample was decanted to eliminate coarse solids. At least 500 mL was allowed to settle in a glass beaker for 15 min and the supernatant was collected. After centrifugation at 3,220 X g for 15 min at 4°C, supernatant was discarded and biomass suspended in the same volume of sterile saline solution (0.8% w/v NaCl), to remove most of environmental inhibitors.

2.2.2 Bacterial isolation

Treated samples were used both for direct isolation and selective enrichment step followed by isolation. For the direct isolation, 100 µl of treated sample was spread in Tryptone Soy Broth with 1.5% w/v agar (TSA; Biokar Diagnostics, France), after serial dilution (10^{-1} to 10^{-7}) with sterile saline solution and incubated at 28°C for 24 h to 48 h. Morphologically different colonies from each sample were picked for isolation. To ensure purity, selected colonies were re-streaked in new TSA plates for 3 consecutive times from an individualized colony. For selective enrichment, 10 mL of biomass sample was incubated in 90 mL sterile wastewater for 7 days, at 28°C 150 rpm. After incubation, an aliquot of enrichment growth was used for isolation in TSA, applying a procedure similar to direct isolation. Two different collections

were set-up: BBC (BioTask Bioremediation Collection) for isolates obtained from environmental samples and CBC (Commercial Bioremediation Collection) for isolates obtained from commercial products.

All isolates were characterized for Gram, cell shape, catalase and oxidase. For Gram test, two methods were used: a staining and a non-staining method. The staining method was executed as described by Gerhardt (1981), using freshly grown cells observed in bright field microscope for cell staining and morphology (rod and cocci). The non-staining method was described by Bucks (1982). Oxidase test was carried out with 1% tetra-methyl-*p*-phenylenediamine dihydrochloride in water solution (Kovács, 1956) and catalase test was applied as described by Taylor and Achanzar (1972).

2.2.3 DNA extraction

A protocol modified from Pitcher *et al*. (1989) was successfully applied for both Gram positive and negative bacteria. A visible loop of cells from a TSA plate was suspended in 1 mL TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and centrifuged (5,200 X g) for 10 min. Washed cell pellet was suspended in 250 µl of lysozyme solution (10 mg/mL lysozyme in TE) and incubated for 2 h at 37°C. Next, 500 µl GES reagent (5 M guanidium thiocyanate, 100 mM EDTA and 0.5% w/v sarkosyl) was added and mixed by inversion. Lysates were cooled on ice for 5 min, then 250 µl cold 10 M ammonium acetate solution added with mixing and held on ice for 10 min. Then, 500 µl chloroform and isoamyl alcohol solution (24:1 v/v) was added and mixed by inversion. After centrifugation (18,000 X g) for 10 min, the supernatant was transferred to a new microtube and equal volume of cold isopropyl alcohol added. The precipitated nucleic acids pellet was centrifuged (18,000 X g) for 10 min and washed with 1 mL 70% v/v ethanol cold solution. Following ethanol evaporation, RNA removal was performed: the nucleic acid pellet was suspended in 500 µl TE with RNase (50 µg/mL RNase in TE) and incubated for 30 min at 37°C. Next, an extraction with chloroform was performed as above and, after centrifugation (18,000 X g; 10 min), the supernatant was transferred to a new microtube and DNA was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of cold absolute ethanol. After centrifugation (18,000 X g; 10 min), DNA pellet was washed with 1 mL 70% v/v cold ethanol solution. After ethanol evaporation, the total DNA pellet was dissolved in 50 µl TE and kept at -20°C until use. Total DNA integrity was checked by agarose gel (1% v/v) electrophoresis in 0.5X TBE buffer (45 mM Tris, 45 mM boric acid and 1 mM EDTA, pH 8.0) at 90 V for 1 h.

2.2.4 Molecular fingerprinting and identification of isolates

To assess the genomic diversity of the isolates, a single primer PCR-fingerprinting approach adapted from Chambel *et al*. (2007) was applied, using two different primers: the M13 minisatellite core sequence (csM13; 5'GAGGGTGGCGGTTCT3') from M13 bacteriophage (Huey and Hall, 1989; Marques *et al*., 2011) and the universal primer PH (5'AAGGAGGTGATCCAGCCGCA3') generally used as the reverse sequence for 16S rRNA gene amplification (Massol-Deya *et al*., 1995). PCR amplifications were performed in a T1 Thermocycler, Biometra, using a total volume of 25 µL and including: 1X reaction buffer, 1 U of TaqDNA polymerase, 0.2 mM of each deoxynucleoside triphosphate, 3 mM MgCl2, 1 mM of primer and 1 µl of DNA extract. All reagents were purchased from Invitrogen, UK. For csM13 primer, PCR cycling conditions were: 94°C for 5 min followed by 40 cycles of 1 min at 94°C, 2 min at 50°C and 2 min at 72°C, plus one additional cycle at 72°C for 5 min. For PH primer, PCR cycling conditions were: 94°C for 3 min followed by 40 cycles of 1 min at 94°C, 2 min at 37°C and 2 min at 72°C, plus one additional cycle at 72°C for 5 min. PCR product profiles were visualized (10 µl) after electrophoresis (90 V for 3 h) in agarose gel (1.2% w/v) 0.5X TBE buffer and staining with ethidium bromide. A 1-kbp DNA Ladder Plus (Invitrogen, UK) was used as a DNA molecular mass marker.

The densitometric profiles of the isolates were compared by dendrogram analysis using the Pearson correlation coefficient, considered an appropriate similarity measure of PCR-fingerprints (Giraffa *et al*., 2000). The unweighted pair group method with arithmetic average (UPGMA) was chosen as clustering method, using the software package BioNumerics version 6.6 (Applied Maths, NV). At least 10% of isolates were randomly selected and analysed in duplicate with each single primer for reproducibility assessment. Furthermore, the molecular diversity among isolates was assessed using Simpson index (*D*) which measures the probability of two non-related isolates, taken from the tested population, belonging to two different genomic types, according to the number of types and isolates for each type (Hunter and Gaston, 1988; Simpson, 1949).

Taxonomical identification of selected isolates was achieved by sequencing of 16S rRNA gene, using pA as forward primer (5'-AGAGTTTGATCCTGGCTCAG-3', positions 8 to 27 in *Escherichia coli* sequence) and 1392R as reverse primer (5'- ACGGGCGGTGTGTRC-3', positions 1392 to 1406 in *E. coli* sequence) for amplification (Lane, 1991). PCR reactions were carried out as described for single primer PCR-fingerprinting. PCR cycling consisted in an initial denaturation step at 94°C for 5 min, followed by 35 cycles (1 min at 94°C, 1 min at

50°C and 1 min at 72°C), and a final extension at 72°C for 3 min. PCR samples were (5 µl) electrophoresed on agarose gel (1% w/v) 0.5X TBE buffer at 90 V for 1 h. PCR fragments were sequenced in GATC Biotech AG laboratory (Cologne, Germany), and species allocation was achieved by homology search in GenBank database at NCBI (National Center for Biotechnology Information), using BLAST algorithm (Altschul *et al*., 1990).

2.2.5 Evaluation of biodegradation potential

The biodegradation potential of all isolates was evaluated by growth assessment in M9 mineral broth medium (Sambrook *et al*., 1989) supplemented with a pollutant as the only carbon source, at different concentrations. M9 medium contained 42.3 mM $Na_2HPO_4.2H_2O$, 22 mM KH_2PO_4, 8.56 mM NaCl, 18.7 mM NH_4Cl, 1 mM $MgSO_4.7H_2O$, 0.1 mM $CaCl_2$, 0.003 mM $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.4 mM H_3BO_3, 0.03 mM $CoCl_2.6H_2O$, 0.01 mM $CuSO_4.5H_2O$, 0.08 mM $MnCl_2.4H_2O$, 0.01 mM $ZnSO_4.7H_2O$ and 0.001 mM $FeSO_4.7H_2O$ (pH 7.0). Tested compounds, obtained from Sigma-Aldrich, included: oleic acid (OA), as a free fatty acid representative, in a concentration of 1% v/v (OA1.0) and 5% v/v (OA5.0); and triolein (TO), as a triglyceride representative, also at 1% v/v (TO1.0) and 5% v/v (TO5.0).

Growth curves were obtained in triplicate for all isolates in each tested condition through automatic microplate reader Bioscreen C (Oy GrowthCurves Ab Ltd, Helsinki, Finland). Assay parameters were set to absorbance at 600 nm, 1 h measurement interval, continuously intensive shaking for 20 seconds, temperature of 25°C for 7 days. Each plate well contained 300 µl supplemented M9 medium inoculated with 10 µl of a cell suspension. The inoculum was previously grown in Brain Heart Infusion broth (BHI; Biokar Diagnostics) at 28°C overnight in 150 rpm shaking, washed and resuspended in sterile saline solution to OD=0.3. The ability of isolates to grow in minimal medium was tested using M9 medium supplemented with 0.6% w/v glucose (GL0.6) as sole carbon source. For each assay, a positive control for inoculum viability with BHI and a negative control with uninoculated M9 medium were also performed.

The absorbance growth curve data from the 168 time points were converted in Area Under Curve (*AUC*), using equation 2.1:

$$AUC_{xi} = \sum_{j=1}^{168} \frac{\left(\frac{OD_{xij} + OD_{xi(j-1)}}{2}\right)}{(T_j - T_{j-1})}$$

Equation 2.1

where AUC_{xi} stands for AUC for isolate x in condition i, OD for Optical Density and T for time (h). For comparison between assays, the Net Area Under Curve ($NAUC$) was calculated using equation 2.2:

$$NAUC_{xi} = AUC_{xi} - AUC_{control\_i}$$

Equation 2.2

where $NAUC_{xi}$ stands for NAUC for isolate x in condition i by removing the Area Under Curve of each conditions' negative control ($AUC_{control}$). This transformation allowed the comparison between growth curves for all isolates in the tested condition. For an integrative selection of the best degraders, the complete dataset of NAUC values (980 values from 196 isolates in five conditions) was examined by Principal Component Analysis (PCA) using NTSys software (Rohlf, 2008).

2.2.6 Biodegradation evaluation

The ability of selected isolates to degrade triolein and oleic acid, separately, were tested in tubes with M9 medium containing 10 g/L of substrate as sole carbon source. For this, inoculum grown in TSA medium at 28°C overnight, washed and suspended in sterile saline solution to OD=0.1, was added to 10 mL M9 medium and incubated at 28°C with constant shaking for 7 days. Degradation was measured by gravimetric quantification of FOG, based on EPA Method 1664 (USEPA, 2010): 1 mL n-hexane extraction and centrifuged for 10 min at 3,220 X g at room temperature. The extraction was repeated to increase yield to over 90% recovery (data not shown). The solvent extracts were evaporated at 50°C for 50 min under 241 mbar pressure, and then oven-dried for 2h at 100°C. The dry weight obtained indicated the amount of oil and grease present in the sample, from triplicates.

Oxygen consumption was also measured for biodegradation assessment with a BOD OxiTop C measuring device (WTW GmbH, Weilheim, Germany). The same substrates (triolein and oleic acid, at 20 g/L) were applied, following manufacturer recommendations. After inoculation to OD = 0.1, 22.7 mL M9 medium supplemented with each substrate as sole carbon source were added to the amber glass bottle. Sodium hydroxide pellets were placed on the bottle holder to absorb any carbon dioxide produced during microbial metabolism. The bottles were sealed tightly, and OxiTop C measurement heads were screwed onto the bottles. The BOD OxiTop instrument follows the diminishing pressure in the bottle, caused by oxygen consumption from gas phase by microbial activity, and the readily absorption of carbon dioxide by sodium hydroxide pellets (Vähäoja *et al.*, 2005). The increasing pressure

can be correlated to biodegradation rate by each isolate in each tested condition. Measurements were carried out for 6 days, at 25°C, automatically by the equipment. Tests were carried out in duplicate, with a negative control to assess any ambiguous oxygen uptake from the substrates.

2.3 Results and discussion

2.3.1 Contaminated sites as a source of degrading microorganisms

A total of 196 isolates were obtained, with 166 collected from environmental samples (BBC collection) and 30 from commercial inocula (CBC collection). BBC isolates were obtained from seven different WWTP industries, namely fish canning activated sludge (31), potato processing activated sludge (19), municipal oxidative lagoon (17), feed industry activated sludge (16), municipal activated sludge (15), metallurgic activated sludge (15) and petrochemical refinery lagoon (14), as well as from a fast food restaurant grease trap (16) and a truck station hydrocarbon separator (23). Based on their general characterization, isolates were clustered into four structural groups (I to IV), namely Gram positive rods (Group I; mostly catalase positive and oxidase negative), Gram positive cocci (Group II; mostly catalase positive and oxidase negative), Gram negative rods (Group III; mostly catalase positive and oxidase negative) and Gram negative cocci (Group IV; mostly catalase positive and oxidase negative). As shown in Figure 2.1, CBC isolates from commercial inocula were mostly Gram positive rod-shaped bacteria, in contrast to BBC collection that was more heterogeneous, with Gram positive bacteria prevailing for most sources.

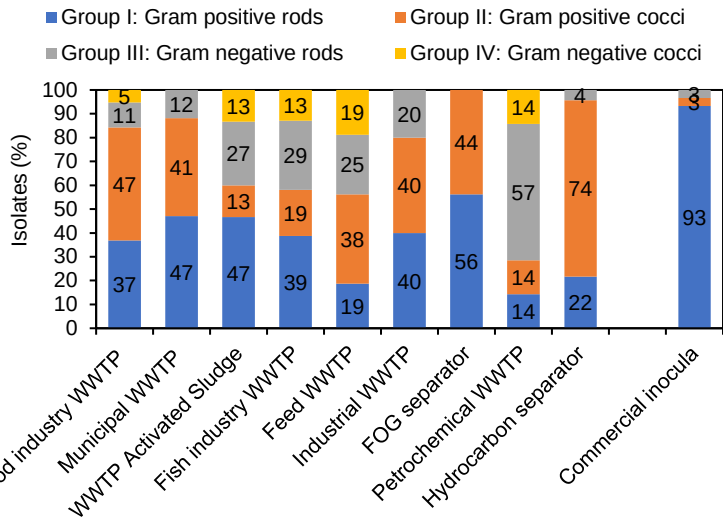


Figure 2.1 – Relative distribution of structural groups of isolates (I to IV) for each isolation source. Each colour represents a different group in terms of ram reaction (positive or negative) and cell shape (rod or cocci).

No statistical association was found between the distribution among structural groups by sample source for natural isolates, suggesting a random distribution over the different environments. Given the origins of the natural samples and the phenotypic discrimination used, such results show the highly diverse microbial species present in WWTP and effluent microbiome. However, the commercial isolates were significantly associated to Gram positive rods (chi-square test; $p<0.001$), in accordance with the already described predominance of strains of Gram positive rods belonging to *Bacillus* spp. in commercial products for bioaugmentation strategies (Tang *et al*., 2012; Tzirita, 2012).

2.3.2 Genomic diversity and biodegradation ability of isolates

Genomic PCR-fingerprints of BBC and CBC isolates were analysed by hierarchical clustering in order to evaluate their genomic diversity and, eventually, detect redundant isolates. Since the average reproducibility of the fingerprints obtained with csM13 and PH primers for all pairs of duplicates was estimated by dendrogram analysis as 90% similarity (data not shown), this value was defined as the cut-off level above which clustered isolates cannot be considered as distinct. To facilitate analysis, the isolates were clustered separately according to structural groups I to IV (Figure 2.2) and the level of 70% similarity was used to assess their relative genomic diversity. A high diversity index ($D \geq 0.91$) was found for all structural groups which could be explained by multiple isolation sites composed of highly dynamic microbial communities (Hu *et al*., 2012).

The biodegradation ability of BBC and CBC isolates was estimated in minimal medium with oleic acid (OA) and triolein (TO) as sole carbon sources (in different concentrations), as well as their growth yield in the presence of glucose. Oleic acid and triolein were selected for biochemical similarity to FOG content found in restaurant effluents (Markossian *et al*., 2000; Nisola *et al*., 2009). The relative NAUC values for each one of these substrates are also represented in Figure 2.2, using a grey shading scale, enabling the cross-analysis between genomic and phenotypic diversity.

Although three clusters of CBC isolates (4, 6 and 7; Figure 2.2a) and two clusters of BBC isolates (11 and 12; Figure 2.2c) were phenotypically coherent, the remaining genomically undistinguishable clusters of isolates showed differences at phenotypic level. Most of this phenotypic heterogeneity seems to be negligible as it corresponds to small NAUC differences (adjacent grey scale levels in clusters 5, 9, 10, 13 and 14), but in some clusters (1, 2, 3 and 8; Figure 2.2a) at least 25% NAUC difference was found for a particular substrate between genomically undistinguishable isolates.

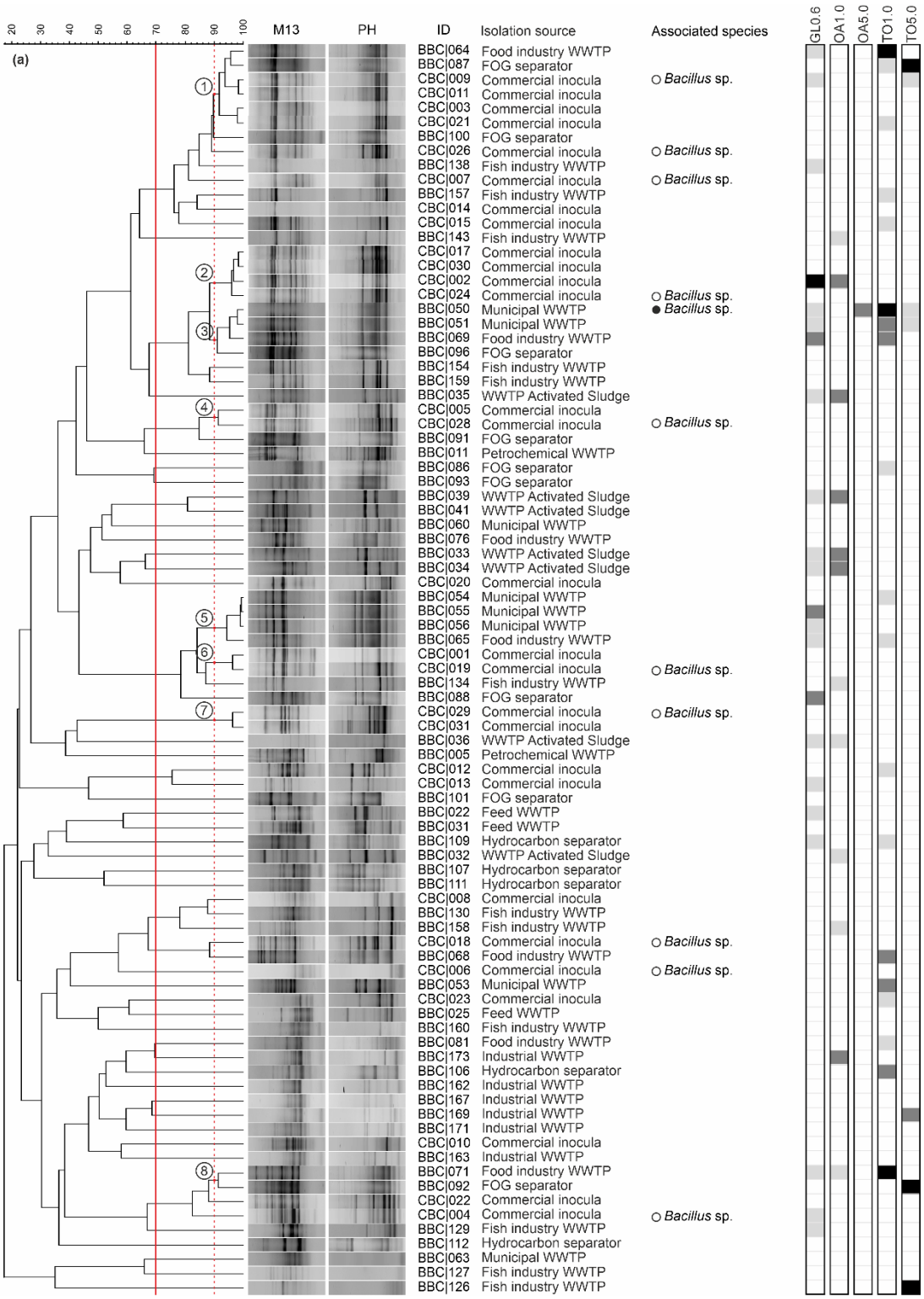


Figure 2.2 – Genomic relatedness of bacterial isolates and growth ability with a sole carbon source in minimal M9 medium. (**a**) Group I: Gram positive rods; (**b**) Group II: Gram positive cocci; (**c**) Group III: Gram negative rods; (**d**) Group IV: Gram negative cocci. The dendrogram was based on PCR-fingerprinting with primers csM13 and PH, using Pearson correlation coefficient as similarity measure and UPGMA as clustering method. The threshold lines for reproducibility and assessment of Simpson's diversity index are represented at 90% and 70% similarity, respectively. Isolate reference number and isolation source is shown (CBC corresponds to isolates from commercial inocula and BBC corresponds to environmental samples). The isolate growth ability is represented by the quarter of relative NAUC where each isolate is positioned in each tested carbon source: glucose (GL, 0.6 g/L v/v), oleic acid (OA, 1 and 5 g/L v/v) and triolein (TO, 1 and 5 g/L v/v). The molecular identification at genus level is also presented for BBC isolates (●) selected by PCA analysis and some CBC isolates (○). Each numbered cluster (1 to 14) correspond to a subgroup of isolates assumed as identical.

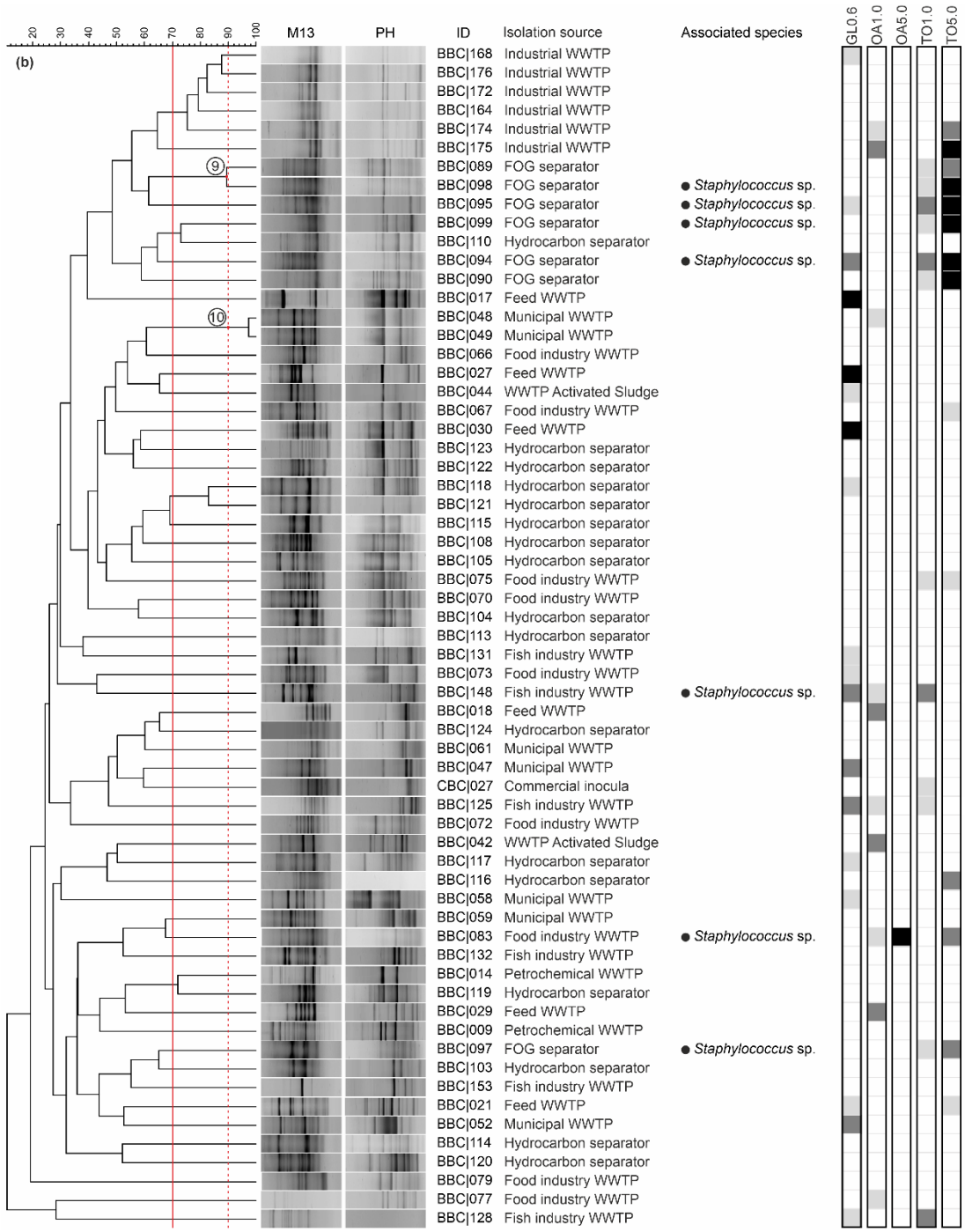


Figure 2.2 (continued)

Since these isolates were recovered from distinct sources, these phenotypic differences may be related with distinct levels of gene expression because of adaptive evolution. Most structural groups contained isolates with NAUC values above 25% for OA and TO consumption, with exception of group IV. However, targeting the top 75% NAUC, group I and II standout with six and seven isolates, respectively, while group III contained only three best degraders while none was found in group IV.

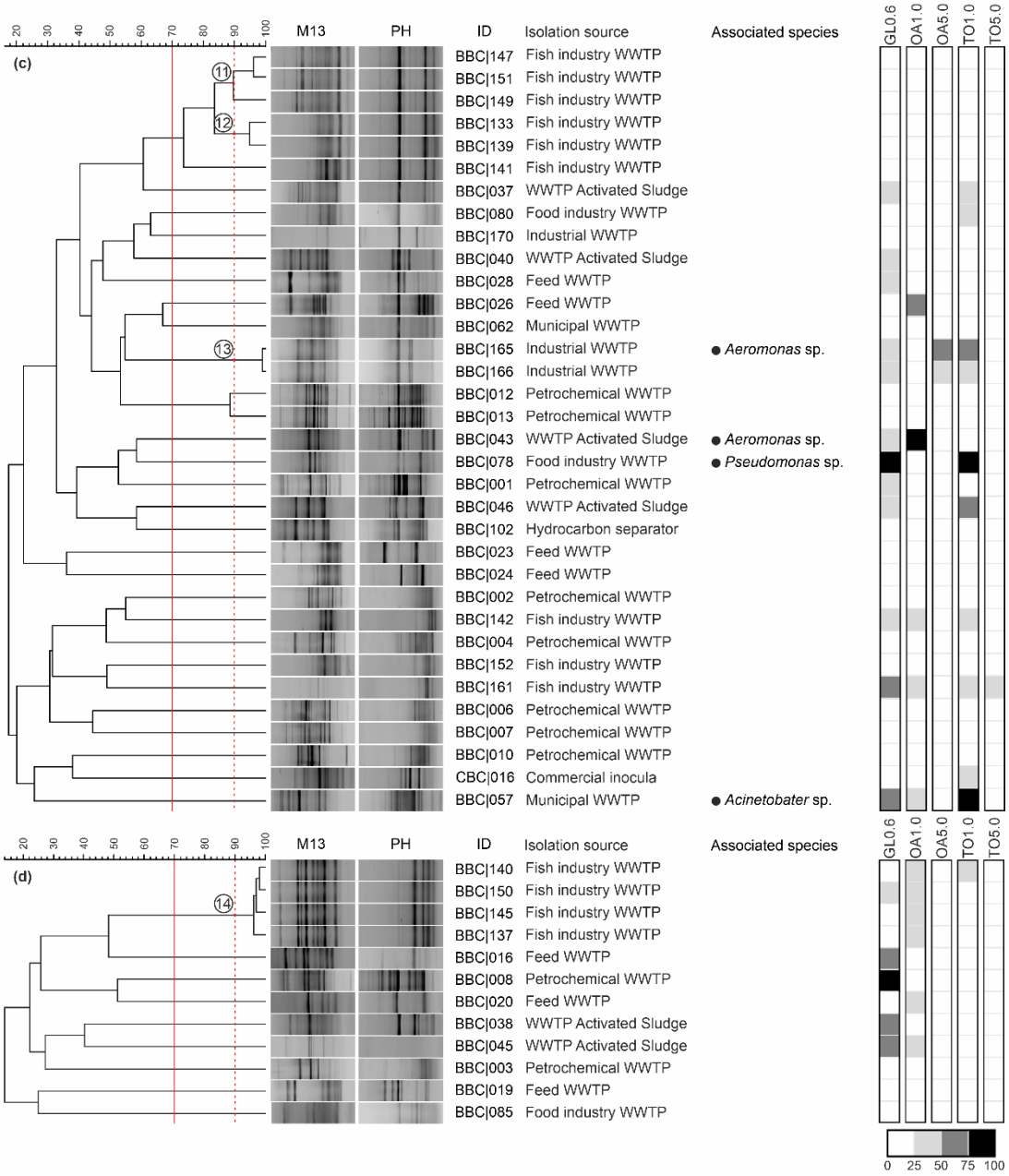


Figure 2.2 (continued)

From these six isolates of group I, two pairs of isolates were genomically undistinguishable, namely, BBC|064 and BBC|087 in cluster 1, and BBC|071 and BBC|092 in cluster 8; the remaining two isolates were genomically distinct from other isolates. Regarding the isolation sources, these isolates were retrieved from FOG contaminated environments, namely, food industry WWTP (2), FOG separator (2), municipal WWTP (1) and fish industry WWTP (1). As for group II best FOG degraders, six isolates were genomically close (BBC|175, BBC|098, BBC|095, BBC|099, BBC|094 and BBC|090), with high affinity for triolein, while the more distant isolate BBC|083, presented better growth with oleic acid. These isolates were retrieved from FOG separator (4), an industrial WWTP (1) and a hydrocarbon separator (1), sources

usually contaminated with FOG residues. Such results show an evident persistency of highly degrading strains in polluted environments, as expected.

Regarding the redundancy of the isolation procedure, 14 small clusters (identified as 1 to 14 in Figure 2.2) where detected, each grouping 2 to 6 isolates genomically undistinguishable. As expected, most of them (clusters 2, 4, 6 and 7 for CBC isolates; clusters 9 to 14 for BBC isolates) correspond exclusively to groups of isolates recovered from the same source, each group probably representing the same strain. The remainder include a mixed group of BBC and CBC isolates (cluster 1) and subgroups of BBC isolates from distinct sources (clusters 3, 5 and 8). Since all these heterogeneous clusters involved WWTP sources that also regularly use commercial inocula, a strong case is made for a common origin amongst them. Furthermore, these results show that species in commercial products are also present in wastewater microbial communities, as previously discussed by Tzirita (2012) for the widespread selection of *Bacillus* and *Pseudomonas* genera in bioaugmentation products. Wastewater microbial community is known to present a low percentage of culturable bacteria on routinely used cultivation media, preventing the complete diversity analysis by previous authors (Wagner *et al*., 1993; Yan *et al*., 2007). However, recent molecular technologies such as high-throughput DNA sequencing has allowed profound understand of wastewater community, regarding different treatment technologies, infrastructures, seasons and operational conditions. At phylum level, Proteobacteria is the most represented bacterial group, accounting for 40 to 65% microbiome diversity (Wells *et al*., 2011; Wang *et al*., 2016), divided mostly by Beta or Gamma and followed by Epsilon, Delta and Alpha classes. Other described phyla were Actinobacteria (6 to 15%), Firmicutes (up to 20%), Bacteroidetes (7 to 11%), and Chloroflexi (up to 8%) (Yan *et al*., 2007; McLellan *et al*., 2010; Wells *et al*., 2011; Hu *et al*., 2012; Ye and Zhang, 2013; Wang *et al*., 2016; Voolaid *et al*., 2017). Although Proteobacteria dominate overall WWTP microbiome, the metagenomic data also shows a small but consistent presence of Firmicutes in such a dynamic and competitive biological system. Without doubts, the metabolic diversity associated to *Bacillus* spp., and the capacity to produce endospores (cell protection gear), facilitate the widespread presence of Firmicutes in wastewater and environmental samples.

Cross-analysis of genomic clustering and biodegradation potential was performed using similarity matrices obtained from genomic fingerprinting and NAUC growth data using Pearson correlation coefficient. When a two-way parameter Mantel test was applied, no significant matrix correlation was found between the genomic similarity of isolates estimated from the molecular fingerprinting and their closeness estimated from growth ability in the

NAUC dataset, for all structural groups (Group I: r=-0.002, p=0.48; Group II: r=0.037, p=0.89; Group III: r=0.189, p=0.88; Group IV: r=0.002, p=0.51). This absence of genomic and metabolic coherency is in agreement with the occurrence of lipase and esterase differential activities in highly diverse bacterial genera (Mongkolthanaruk and Dharmsthiti, 2002; Loperena *et al*., 2009; Prasad and Manjunath, 2011; Emeer *et al*., 2014), encompassing Gram positive rods and cocci (*Bacillus* spp. and *Staphylococcus* spp.) as well as Gram negative rods (*Serratia* spp.).

2.3.3 Selection of FOG native biodegraders

Biodegradation screening in microplate assays has been widely applied for biodegraders selection towards different pollutants, as nitrophenolic compounds, azo-dyes, plastics or benzotriazole (Tvrzová *et al*., 2006; Bailes *et al*., 2013; Herzog *et al*., 2014; Lu *et al*., 2015; Meerbergen *et al*., 2018). The NAUC parameter, which depends on both growth rate and maximum cell number, allowed the comparison between all isolates in all conditions, with a normalized value. A Principal Component Analysis (PCA) of NAUC data obtained for the 196 isolates in overall tested conditions (980 growth curves with 168 time points each) was used to select in an integrative approach the most promising isolates. In Figure 2.3 are represented the biplot involving the first two PCs, which retained 52.6% of initial variance. As seen in Figure 2.3 correlation circle, PC1 was positively associated to growth with glucose as sole carbon source (GL0.6, r = 0.683), oleic acid 5% v/v (OA5.0, r = 0.574) and triolein 1% v/v (TO1.0, r = 0.727), and PC2 was positively associated to oleic acid 1% v/v (OA1.0, r = 0.702), and negatively related to triolein 5% v/v results (TO5.0, r = -0.652).

All selected natural isolates and some commercial isolates were identified by 16S rRNA sequencing (Figure 2.2), by BLAST algorithm based on a sequence ranging from 495 bp to 1,322 bp. As shown in Figure 2.2, most isolates from the commercial inocula belong to *Bacillus* spp., as expected from producers datasheet referring the presence of 8 different *Bacillus* spp. In fact, several commercial inocula for bioaugmentation are described as mainly composed by strains of endospore-forming genus, for its low biosafety level, increased viability and extended shelf-life (Brooksbank *et al*., 2007; Tang *et al*., 2012; Tzirita, 2012).

The exploratory approach by PCA using NAUC, allowed to analyse 196 isolates grown in five different conditions. The data showed that most isolates from commercial inocula were clustered together near the origin points (Figure 2.3), suggesting lower biodegradation ability compared to overall distribution.

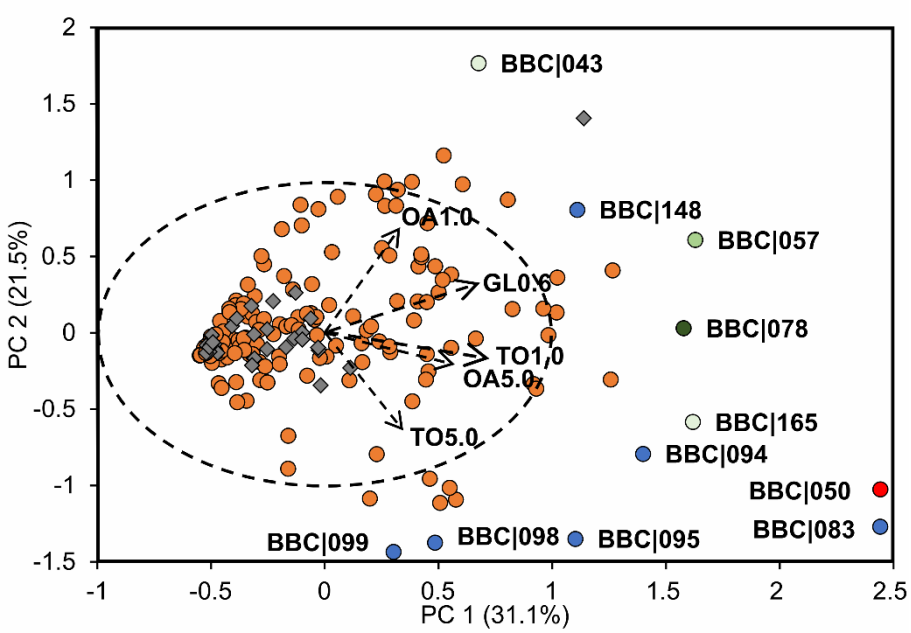


Figure 2.3 – Principal Component Analysis of Net Area Under Curve data obtained for the 196 isolates in minimal medium with different carbon sources. PC1 and PC2 refer to the first two principal components, accounting for 31.1% and 21.5% of total variance, respectively. The correlation circle including all variables is depicted. GL0.6: glucose 0.6 g/L v/v; OA1.0 and OA5.0: oleic acid 1 and 5 g/L v/v; TO1.0 and TO5.0: triolein 1 and 5 g/L v/v. CBC isolates, from commercial inocula, are represented by grey diamonds and BBC isolates, from environmental sources, by coloured circles. Orange circles: unselected BBC isolates; red circle: selected BBC isolate from group I (*Bacillus* sp.); blue circles: selected BBC isolates from group II (*Staphylococcus cohnii*); green circles: selected BBC isolates from group III (*Pseudomonas* sp., dark green; *Acinetobacter* sp., green; *Aeromonas* spp., light green).

This could result from lack of metabolic pathway for some of the tested compounds, activator requirements or limited growth capacity in the tested conditions. Furthermore, commercial isolates did not globally exceed natural ones, indicating that selection of new strain could lead to new bioaugmentation products with higher biodegradation efficiency. Since all PCs axes represent a gradient of higher growth ability towards its positive (PC1 and PC2) or negative (PC2) side, best degrading isolates were positioned further from the centre main cluster (Figure 2.3). Therefore, isolates BBC|043, BBC|148, BBC|057 and BBC|078 were selected for biodegradation of free fatty acids at low concentration; BBC|165, BBC|094, BBC|050 and BBC|083 were associated to removal of free fatty acids at high levels and triglycerides at low levels; and BBC|095, BBC|098 and BBC|099 showed increased ability to bioremediation of high concentration of triglycerides, totalizing 11 selected isolates with the overall highest biodegradation potential. The integrative approach allowed to select the best degraders for each tested substrate and those with overall good growth results, namely, BBC|148 and BBC|165. Analysis of isolates presenting over 75% NAUC for only one condition (Figure 2.2) would lead to the additional selection of BBC|064 and BBC|071 (for TO1.0) and BBC|087, BBC|090, BBC|092, BBC|126 and BBC|175 (for TO5.0). Nevertheless, given the

main goal for new bioremediation strategies, isolates showing multiple growth abilities could be more resilient to colonize highly competitive environments and integrate WWTP microbiomes.

Regarding the 11 selected isolates, one was a Gram positive rod (Group I), six were Gram positive cocci (Group II) and four were Gram negative rods (Group III). However, none was from Group IV (Gram negative cocci), corroborating the low biodegradability ability found it this group. The selected isolate from Group I (BBC|050) was part of cluster 3 (Figure 2.2a), a genomically homogeneous but phenotypically heterogenous group of isolates as already referred. Selected isolates from Group II encompass two genomically unrelated ones (BBC|083 and BBC|148) and a closer group which includes BBC|098, BBC|095, BBC|099 and BBC|094, all presenting high TO potential degradation ability. Also, BBC|098 was part of cluster 9 (Figure 2.2b), in association with BBC|089, with similar phenotypic response and common origin. In fact, the main group was retrieved from FOG separator samples while the selected two isolates were from a fish industry WWTP (BBC|148) and a food industry WWTP (BBC|083), sites also polluted with FOG residues. Among Group III selected isolates, BBC|165 was associated to BBC|166 in cluster 13 (Figure 2.2c), an isolate with lower phenotypic response for OA and TO growth. Given the common sample origin (industrial WWTP), the heterogenic response could indicate natural adaptive evolution in real WWTP microbiome. The selected isolates were mostly retrieved from FOG separator samples (four isolates), while the remaining ones have a more diverse origin comprising municipal and food industry WWTP (two isolates each) and activated sludge, fish industry and industrial WWTP (one isolate each). A FOG separator usually maintains a highly contaminated wastewater flow with variable hydraulic retention time, a defying environment for microbiological establishment. Given this, the selected isolates from such a harsh location present a high potential for novel bioremediation products for FOG contaminated environments.

Considering the identification of selected isolates, all genera have been previously associated to the biodegradation of the tested pollutants. Fang and colleagues (2006) showed lipase production associated to a *Staphylococcus* sp. isolate, as inferred from growth with triglyceride (triolein) as sole carbon source by *Staphylococcus* spp. isolates BBC|083, BBC|094, BBC|095, BBC|098, BBC|099 and BBC|148. *Aeromonas* spp. isolates BBC|043 and BBC|165 showed lipolytic ability, as already reported for this genus (Pemberton *et al*., 1997; Sarmurzina *et al*., 2013; Emeer *et al*., 2014). Previous studies also associate *Bacillus* spp. to lipid degradation (Markossian *et al*., 2000; Brooksbank *et al*., 2007; Tang *et al*., 2012; Tzirita, 2012; Tzirita *et al*., 2018; Tzirita *et al*., 2019), as observed with *Bacillus* sp. isolate BBC|050.

Other authors reported *Acinetobacter* spp. associated to lipid-rich and dairy wastewater biodegradation (Loperena *et al*., 2009; Mongkolthanaruk and Dharmsthiti, 2002; Gao *et al*., 2019) as seen with *Acinetobacter* sp. BBC|057. Also, *Pseudomonas* spp. have been applied in consortia for wastewater bioremediation strategies (Shon *et al*., 2002, Sarmurzina *et al*., 2013) for its metabolic plasticity, supporting the results obtained with *Pseudomonas* sp. BBC|078. In fact, Tzirita and colleagues (2019) obtained a successful cooperative bioremediation process by applying both *Bacillus* spp. and *Pseudomonas* sp. to enhance butter (1% w/v) and olive oil (1% v/v) lipid content removal. Even though all BBC isolates belong to genera already associated to lipids degradation, their origin from polluted environments ensures a higher resilience to cope with the constantly evolving dynamics of such sites and constitutes an added value to the design of novel bioaugmentation products.

2.3.4 Biodegradation evaluation

The biodegradation capacity of the selected isolates was measured in M9 growth medium supplemented with 10 g/L of oleic acid or triolein as sole carbon source (Figure 2.4). After a 7 days treatment at 28ºC, best degraders were BBC|043 and BBC|148, with 37.9% and 21.2%, for oleic acid, and 17.1% and 19.1% triolein removal, respectively. Some isolates showed no degradation capacity for both substrates (BBC|050 and BBC|095) or triolein (BBC|057, BBC|098 and BBC|165) in these assays, with final FOG content within the abiotic degradation (5.1% for oleic acid and 6.1% for triolein). The abiotic degradation may be caused by recovery limitations from FOG quantification method, as noted by EPA report, ranging from 83% to 101% (USEPA, 2010).

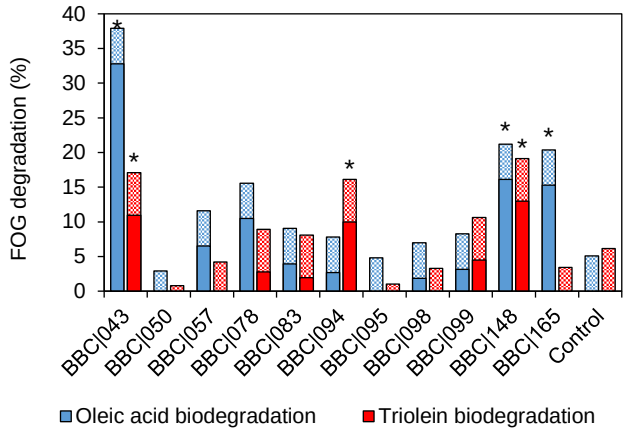


Figure 2.4 – Oil and grease removal (FOG) by eleven selected isolates after 7 days treatment in M9 mineral medium supplemented with 10 g/L substrate as sole carbon source: oleic acid (blue bars) or triolein (red bars). Control: non-inoculated M9 medium plus substrate. Data are means of experiments performed in triplicates, with abiotic degradation represented by dotted bars for each tested substrate. (*Two tailed *T* test with control condition, $p < 0.05$).

Overall, oleic acid was more easily metabolized by selected isolates, with exception to BBC|094, which showed a higher triolein biodegradation. The level of lipidic content removal achieved with the selected isolates (Figure 2.4) was in the range obtained by Brooksbank and colleagues (2007), when using enriched nutrient medium and more complex carbon sources (lard, soya, sunflower and rapeseed oils) for 21 days assay with commercial product. Other authors reached higher FOG degradation, ranging from 55% to 94.5%, when applying higher initial inoculum (more than 10^8 CFU/mL) and mineral medium or wastewater supplemented with complex carbon sources, in 7 days assays (Shon *et al*., 2002; Sarmurzina *et al*., 2013) or 12 days assays (Mongkolthanaruk and Dharmsthiti, 2002; Prasad and Manjunath, 2011; Emeer *et al*., 2014). Matsumiya and colleagues (2007) reported a *Burkholderia* sp. DW2-1 capable of degrade up to 77% FOG content after 48 h at 30ºC using beef tallow as substrate in synthetic wastewater medium. Gao and colleagues (2019) described three bacterial isolates (two *Acinetobacter* spp. and one *Kluyvera* sp.) able to remove over 90% lipid content after 72 h at 30ºC, from edible oil at 2% (v/v) in mineral medium. *Acinetobacter soli* SP2 was also reported to efficiently degrade the emulsifier Polysorbate 80, with 50% removal after 8 h growth (Nguyen, 2018), supporting the lipolytic ability of bacteria from *Acinetobacter* genus. Differences in nutrient basal medium, temperature, substrate (pure and/or complex) and initial inoculum will affect biodegradation ability of isolates. The disparity found in biodegradation values can be explained by differences in lipid content measurement methods (gravimetric or chromatography), in cell growth monitoring (total cell count or absorbance), growth condition (time, temperature, pH and agitation), tested substrates (single compound or mixed) and media (complete or mineral media). However, the selected isolates showed significant enhanced FOG biodegradation (by two-tailed *T*-test, $p<0.05$) in both pure substrates (Figure 2.4), evidencing the success of the screening and selection approach applied for the initial 196 isolates.

Biodegradation rate was followed by respirometry approach for best degraders (BBC|043 and BBC|148), and 3 others with different responses for each substrate (BBC|083, BBC|095 and BBC|165), as shown in Figure 2.5. As expected, BBC|043 and BBC|148 presented the highest oxygen consumption when grown solely with oleic acid or triolein. Oxygen uptake correlates to biodegradation rates for aerobic metabolism of substrates (Vähäoja *et al*., 2005), allowing an indirect monitorization parameter of microbial growth. The respiratory results confirmed the previous biodegradation assay, with BBC|043 and BBC|148 achieving the highest oxygen consumption rates (oleic acid: 2,598 and 5,597 mg O_2/L.day; triolein: 841 and 3,188 mg O_2/L.day). In both cases, BBC|043 required a longer adaptation phase to initiate

exponential growth, 4 days for oleic acid and 1 day for triolein, while BBC|148 growth response began after 7 h for both substrates. The remaining three isolates presented similar responses, with BBC|165 achieving a higher final oxygen consumption in both substrates (OA: 989 mg O_2/L; TO: 452 mg O_2/L).

A longer adaptation phase in FOG biodegradation was detected for BBC|043 isolate by respirometry assay (Figure 2.5), which could result from stress impact caused by oleic acid (Desbois and Smith, 2010). In fact, oleic acid affects Gram negative species, as discussed by Hinton and Ingram (2000), where 10% w/v oleic acid showed small cellular viability impact for *Enterobacter cloacae*, *Staphylococcus lentus* and *Salmonella typhimurium*, while *Escherichia coli*, *Enterococcus faecalis*, *Listeria monocytogenes* and *Pseudomonas aeruginosa* species were severely affected.

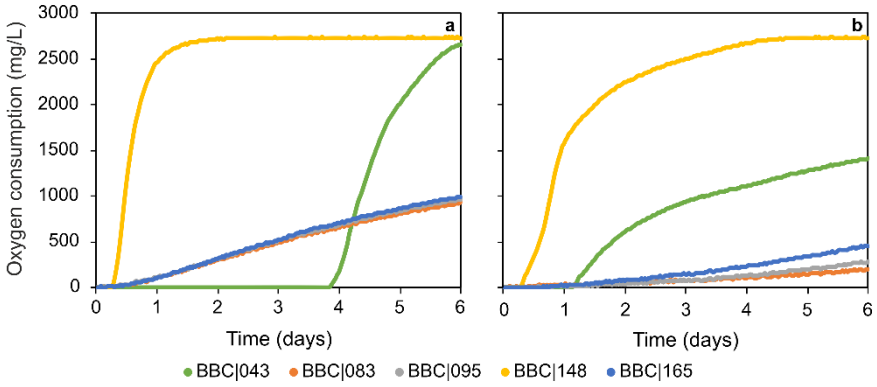


Figure 2.5 – Oxygen consumption over 6 days at 25ºC in M9 mineral medium supplemented with 20 g/L substrate as sole carbon source: oleic acid (**a**) and triolein (**b**).

On the other hand, the adaptation phase observed for triolein could be explained by enzymatic machinery production. In lipids degradation pathway, first degradation step is performed by lipase, to yield fatty acids and glycerol as substrates for microbial growth, which may be a growth limiting process since such enzymes are usually not constitutively produced (Becker, 2010). Given the source and taxonomic diversity of these new isolates, their lipases could present suitable features for different applications. In fact, lipases present broad range application in different areas: in the food sector, for fat monitoring in products, for flavour enrichment, to improving baking process and tea leaves preparation; in other sectors: for biodiesel manufacturing, innovative beauty products, new detergents, and in paper industry for deinking recycled paper (Nawal *et al*., 2019).

2.4 Conclusions

In summary, isolates from commercial products presented low taxonomic and phenotypic diversity, unlike natural isolates. Given the highly diverse and dynamic microbiome in WWTP, such low diversity could hinder microbial response and require acclimatization steps for new environments in real scale applications. To ensure bioaugmentation strategies success and viability, exogenous strains must thrive when exposed to real wastewater conditions and its natural microbiome. Results suggested that improvement of bioaugmentation products is possible through screening for more efficient and fast responsive bacterial strains from environmental sources. Biodegradation screening by an integrative data analysis, allowed the selection of 11 promising isolates for bioaugmentation strategies. A high diversity was observed among selected isolates, including *Bacillus* sp., *Aeromonas* spp., *Acinetobacter* sp., *Pseudomonas* sp. and *Staphylococcus* spp., species previously associated to lipid degradation. Biodegradation capacity for the tested isolates was confirmed by two different strategies, gravimetric removal quantification and respirometry measurement.

The two best degraders BBC|043 and BBC|148, identified by 16S rRNA gene sequencing as belonging to *Aeromonas* sp. (GenBank number: MK687553) and *Staphylococcus cohnii* (GenBank number: MK687554), were deposited in the University of Coimbra Bacteria Culture Collection under the accession numbers UC-CCB 65 and UC-CCB 66, respectively. The selected isolates present no genetic modifications and good biodegradation ability for several pollutants, and have been retrieved from WWTP, the expected location for real-scale removal of such pollutants. These isolates showed great features for future bioaugmentation products, to promote lipids degradation, both by *in situ* and *ex situ* approaches.

Chapter III - Adaptive evolution of wastewater microbiome towards hydrocarbon and lipid enhanced bioremediation

3.1 Introduction

Since the industrial revolution, modern society has grown by exploring our natural resources at a global scale. These industrial processes release chemicals into the air, water and soil, whether from production, transportation, combustion, or accidental release. Most of these chemicals are quite recalcitrant and natural processes cannot keep up with the releasing rate, creating a pollution accumulation phenomenon. The European Commission (EC) and the Environmental Protection Agency (EPA) have designated the most dangerous pollutants in the environment in recent and future years in the Priority Substance List. In the top of this list are polycyclic aromatic hydrocarbons (PAH), produced by chemical and petrochemical industries, with harsh wastewater effluent contaminated with PAH (Cerniglia 1992), as well as combustion engine exhaust gases, as the main source for PAH air contamination (Khalili *et al*., 1995). PAHs are suspected mutagens, with toxic effects on human health and reduced treatability in biological WWTP systems (European Commission, 2001; Environmental Protection Agency, 2012). The most applied engineering solution for PAH treatment rely on landfill deposit, a slow and inefficient process, presenting several potential hazardous consequences as water leachate and underground water contamination.

Our industrialized food and feed process present similar problems with the release of other recalcitrant pollutants. Most problematic are FOG residues, as already reported in Chapter 2, specifically saturated fats with low water solubility which form deposits in sewage systems (Keener *et al*., 2008). Such problematic pollutants are still of concern for WWTP operators given the limitation for biological treatment. Also, engineering solutions to high FOG content wastewater target physical removal of FOG from wastewater, whether by density separation, as a FOG separator, or by forced flotation and a skimmer for oil collection. However, such approach does not eliminate these compounds, only concentrates the problem for further treatment.

Adaptive evolution experiments (AEx) can be used to enhance microbial strains for multiple purposes, allowing to harness further biological activities. Microorganisms are well suited for such strategy, given the short generation time, allowing the accumulation of spontaneous mutations in an appliable timeframe, and also the ability to maintain a large population in controlled conditions and store it for later examination (Elena and Lenski, 2003). Natural evolution is steered by environmental changes, competition and isolation of small populations, which can be mimic in laboratory conditions. For AEx, isolated microorganisms or consortia are expected to evolve under controlled scenarios, mimicking natural stress situations, to accelerate the evolution process in order to obtain improved strains, presenting

enhanced features over the initial population. As Figure 3.1 shows, different methodologies have been used, from consecutive colony transfer from solid growth medium, to serial transfer from flask growth and also chemostat controlled growth (Jeong *et al*., 2016). The plate colony transfer is limited to solid medium growth conditions, which do not mimic most natural conditions and usually rely on low population size. The chemostat culture is a continuous stress inducer system, were a population is kept and forced to evolved by direct competition, in the same location. It requires specific control and equipment to maintain such experiment on operation over long periods of time to give rise to new strains. The serial transfer method presents a simpler approach, which allows the use of complex carbon source or different environmental conditions, limited by the number of flasks to keep over time. In AEx, each new cycle represents an evolutionary bottleneck, when only part of the biomass is transferred for the new cycle, leading to selective wash-out of slow growing strains.

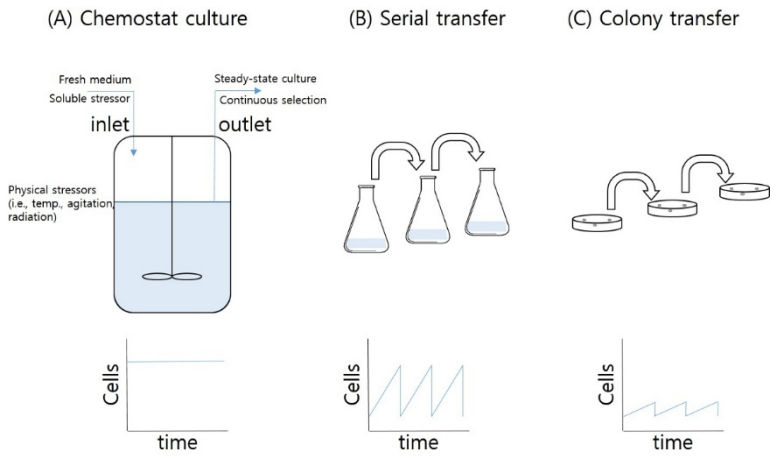


Figure 3.1 – Methods of adaptive evolution experiments (AEx). (A) Chemostat; (B) serial transfer; (C) colony transfer. The top figures illustrate the concept of the methods for AEx and the bottom figures illustrate the number of cells that grow during AEx. Extracted from Jeong *et al*., 2016.

In initial studies from the 80's, molecular evolution was assessed by gene modification and modulated its impact in overall metabolic ways. Today, genomic advances have provided tools for whole genome sequencing approach to determine AEx effect on evolved strains, both at nucleotide as well as whole genome level. This helped to establish grounding evolutionary theories to the actual molecular and metabolic impact and mechanistic bases of evolution at laboratory experiments (Conrad *et al*., 2011).

In the present work, four different experiments were carried out with different insoluble pollutants as sole carbon source in a mineral media. These experiments present a harsh environment, with alternative carbon source (a pollutant) as the evolutionary stress, at low

mesophilic temperature. The pollutants used were anthracene and phenanthrene, proxy for PAH pollution; lubricant mineral oil, a proxy for aliphatic hydrocarbons; glyceryl tristearate, a proxy for solid saturated triglycerides. Both anthracene and phenanthrene are short PAHs, with 3 aromatic rings, linear and angled, respectively. Although sharing most chemical and physical properties, the ring fusion site in phenanthrene makes it more stable and harder to degrade, both chemically and biologically. Gutman and Stanković (2007) referred that phenanthrene molecular conformation grants a shield for bond disruption, requiring more energy to disrupt it and lead to molecular instability. Moreover, both compounds have been used as proxy for higher PAH molecules, both linear (as tetracene and pentacene, with four and five rings, respectively) and angled (chrysene and benzo[α]anthracene, benzo[α]pyrene, with four and five rings), given the similar chemical properties and low environmental toxicity (Phale *et al*., 2019; Samanta *et al*., 1999; Wornat *et al*., 1992). The glyceryl tristearate (also referred as stearin or tristearate) is a triglyceride, composed by a glycerol residue with three molecules of stearic acid, a saturated fatty acid with 18 carbons. Most common fatty acids in human diet are medium sized molecules, from 16 to 22 carbon length, both saturated and unsaturated (Table 3.1). Glyceryl tristearate is obtained from animal fat, specially processed beef, mutton and cod oil, highly insoluble in water. Given the chemical similarities among common fatty acids in human diet, and the prevalence of animal fat, tristearate was tested has a proxy for triglycerides, to obtain enhanced novel FOG-degradative bacteria for bioaugmentation.

Table 3.1 – Characteristics of most common fatty acids in human diet. From White (2009)

Name	*Number of carbons*	*Fatty acid saturation state*	*Common source*
Palmitic acid	16	Saturated	Palm oil
Stearic acid	18	Saturated	Animal fat
Oleic acid	18	Monounsaturated	Olive oil
Linoleic acid	18	Polyunsaturated	Safflower oil
Linolenic acid	18	Polyunsaturated	Soybean oil
Arachidonic acid	20	Polyunsaturated	Meat, dairy
Eicosapentaenoic acid	20	Polyunsaturated	Fish oil
Docosahexaenoic acid	22	Polyunsaturated	Fish oil

The lubricant mineral oil, from a commercial mixture of aliphatic hydrocarbons, was the only liquid pollutant applied in this work. Lubricant mineral oil contains a mixture of hydrocarbons with different size and structure, derived from crude oil and/or synthetically produced from coal, natural gas or biomass. Usually, chemical composition varies from batch to batch, since specifications are expressed in terms of viscosity or thickness, according to applications,

given the wide variety of industrial and domestic uses. The application of such compounds has led the EFSA (*European Food Safety Authority*) to alert the possible contamination of food with mineral hydrocarbons, derived from food packaging materials, additives, processing aids and lubricants. Furthermore, these compounds can be considered genotoxic carcinogens (by interfering with DNA replication), accumulate in human tissues and cause adverse effects in the liver, while in some EU countries, low- medium- viscosity mineral oils are authorised for use as food additives (Alexander *et al*., 2012; EFSA, 2009). Given all their features, the selected pollutants represent insoluble solid PAHs, triglycerides and liquid hydrocarbons, applied in a stratified growth media.

The AEx approach has been successfully applied in single strain improvement for a specific trait (pH tolerance, toxic compound, temperature), or applied in population dynamic competition (Fong *et al*., 2005; Wallace-Salinas *et al*., 2013; Horinouchi *et al*., 2015; Shui *et al*., 2015; Li *et al*., 2019; Mo *et al*., 2019). In the present work the starting community comes from a WWTP microbiome, a quite complex and variable population. Such variability has been associated to wastewater load, infrastructure, seasonality, and WWTP operation parameters (Hu *et al*., 2012; McIlroy *et al*., 2015; Voolaid *et al*., 2017). The major difficulty in studying such environment arises from the low bacterial diversity recovered from culture-dependent methods when compared to culture-independent. In fact, most recent NGS (*Next Generation Sequencing*) methods have indicated the presence of relevant site-specific groups associated to different infrastructures in WWTP. The microbial profiling has been successfully applied for WWTP and studied by several groups, giving rise to specialized public databases such as **MiDAS** (***Mi****crobial* ***D****atabase for* ***A****ctivated* ***S****ludge*) intending to "*summarize all the knowledge about the physiology and ecology of the important microorganisms present in wastewater systems*" (www.midasfieldguide.org/guide). The increase in molecular data shows the importance of specialized databases for analysis of genomic data, to reduce data bias and to allow discussion and analysis of relevant data (Fan *et al*., 2014). Understanding population dynamics and the most relevant microbial groups can aid WWTP operator to improve biological treatment, while allowing the selection of bacterial species most fitted for this niche. Regarding bioremediation, culture-dependent approaches are fundamental to select relevant microorganisms, with potential applications in novel bioaugmentation products to improve biological wastewater treatment.

3.2 Materials and Methods

3.2.1 Adaptive evolution experiments

Adaptive evolution experiments (AEx) were performed in mineral M9 medium (Sambrook *et al*., 1989, as in Chapter 2) with 2% w/v substrate as sole carbon source for selective pressure. The carbon substrates represented different pollutants: polycyclic hydrocarbons (ANT, anthracene and PHE, phenanthrene), aliphatic hydrocarbons (MiO, lubricant mineral oil) and triglycerides (GTS, glyceryl tristearate). A WWTP sludge sample served as inoculum, and was kept for 110 cycles, each consisting of a 7-days incubation (continuous shaking of 150 rpm, at 16°C) after 1% inoculum from previous cycle. Given the presence of particulate material (insoluble solid substrates), microbial growth was monitored by total cell count in a Neubauer chamber. For data analysis, the generation number was calculated based on Equation 3.1:

$$g_i = \frac{ln\, N_i - ln\, N_0}{ln\, 2}$$

Equation 3.1

where g_i stands for the number of generations (g) in each cycle i, N_i for cell count in i cycle and N_0 for initial cell count at i cycle start.

3.2.2 Isolation of microbial degraders with selective media

Several AEx cycles were screened in selective solid growth media to isolate microbial degraders. For this, 100 µl of AEx sample was incorporated in selective media, previously described by Atlas (2010). The selective media was applied according to physical state of pollutant: Atlas Oil Agar medium for liquid (lubricant mineral oil) and Oil Agar medium for solid-state compounds (anthracene, phenanthrene and glyceryl tristearate). The Atlas Oil Agar contained 1.5% w/v agar, 7.3 mM KH_2PO_4, 5.7 mM K_2HPO_4, 12.7 mM NH_4NO_3, 0.8 mM $MgSO_4.7H_2O$, 0.3 mM $FeCl_3.6H_2O$ and 0.1 mM $CaCl_2.2H_2O$, also referred to as Bushnell-Haas agar (Bushnell and Haas, 1941), supplemented with 20 mL/L of mineral oil (MiO). The Oil Agar Medium had 1.5% w/v agar, 171.1 mM NaCl, 12.7 mM NH_4NO_3, 4.2 mM $MgSO_4.7H_2O$, 4 mM K_2HPO_4, 2.2 mM KH_2PO_4, supplemented with 1% w/v of oil powder. Oil powder was prepared by dissolving 10 g of substrate in 30 mL of diethyl ether and 10 g of silica gel, then allow ether to evaporate. Also, general growth media (TSA) was used for direct isolation by spreading. Plates were incubated at 28°C for 2-4 weeks until visible colonies were observed and picked. Isolates were included into BioTask Bioremediation

Collection (BBC) after phenotypic characterization for gram, cell shape, catalase and oxidase, following the methodology described in Chapter 2.

3.2.3 DNA extraction

A protocol modified from Pitcher *et al*. (1989) was successfully applied for both bacteria and yeast isolates. A visible loop of cells from a TSA plate was suspended in 1 mL TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8,0) and centrifuged (5,200 X g) for 10 min. Washed cell pellet was suspended in 250 µl of lysis buffer (50 mM Tris, 250 mM NaCl, 50 mM EDTA, 0.3% w/v SDS and 0.5% w/v sarkosyl, pH 8.0) and 100 µl glass beads (425 – 600 µm). After a 2 min shaking in vortex, for mechanical disruption, the lysate was incubated for 30 min at 65°C, followed by another 2 min shaking in vortex. Next, 250 µL GES reagent (5 M guanidium thiocyanate, 100 mM EDTA and 0.5% w/v sarkosyl) was added and mixed by inversion. Lysates were cooled on ice for 10 min, then 125 µL cold 10 M ammonium acetate solution added, mixed by inversion and held on ice for another 10 min. Then, 500 µL chloroform and isoamyl alcohol solution (24:1 v/v) was added and mixed by inversion. After centrifugation (18,000 X g) for 10 min, the supernatant was transferred to a new microtube and added equal volume of cold isopropyl alcohol. The precipitated nucleic acids pellet was centrifuged (18,000 X g) for 10 min and washed with 1 mL 70% v/v ethanol cold solution. Following ethanol evaporation, RNA removal was performed: the nucleic acid pellet was suspended in 500 µL TE with RNase (50 µg/mL RNase in TE) and incubated for 30 min at 37°C. Next, an extraction with chloroform and isoamyl alcohol solution was performed as above and, after centrifugation (18,000 X g; 10 min), the supernatant was transferred to a new microtube and DNA was precipitated by adding 1/10 volume of cold 3 M sodium acetate (pH 5.2) and 2.5 volumes of cold absolute ethanol. After centrifugation (18,000 X g; 10 min), DNA pellet was washed with 1 mL 70% v/v cold ethanol solution. After ethanol evaporation, the total DNA pellet was dissolved in 50 µL TE and kept at -20°C until use. Total DNA integrity was checked by agarose gel (1% v/v) electrophoresis in 0.5X TBE buffer (45 mM Tris, 45 mM boric acid and 1 mM EDTA, pH 8.0) at 90 V for 1 h.

3.2.4 Molecular fingerprinting and representative strains

To assess the genomic diversity and allow genomic discrimination between isolates, single primer PCR-fingerprinting approach was applied (Chambel *et al*., 2007), using three different primers: the M13 minisatellite core sequence (csM13) from M13 bacteriophage (Huey and Hall, 1989; Marques *et al*., 2011), the universal primer PH generally used as the reverse sequence for 16S rRNA gene amplification (Massol-Deya *et al*., 1995) and the microsatellite

$(GTG)_5$ initially described for human genomic individualisation and paternity test (Schafer *et al*., 1988). For PH primer, amplifications were performed in a T1 Thermocycler, Biometra, using a total volume of 25 µL and including: 1X reaction buffer, 1 U of TaqDNA polymerase, 0.4 mM of each deoxynucleoside triphosphate, 3 mM $MgCl_2$, 2 µM of primer and 1 µl of DNA extract. For PCR, cycling conditions were: 94°C for 3 min followed by 40 cycles of 1 min at 94°C, 2 min at 37°C and 2 min at 72°C, plus one additional cycle at 72°C for 5 min. Further steps were followed as previously described in Chapter 2. Dendrogram analysis of densitometric profiles, based on Pearson correlation coefficient as association measure and UPGMA as clustering method (software package BioNumerics version 6.6; Applied Maths, NV), allowed to establish genomic similarity between isolates from different AEx, isolation cycle and selective media. Isolates with similarity of at least 90%, from the same AEx, and presenting a congruent phenotypic group for other features (oxidase and catalase), were considered undistinguishable and were merged into a single cluster (data not shown). From there, each cluster was represented by a strain corresponding to the isolate from the newest cycle.

The molecular diversity among the BBC isolates was assessed using the Simpson index (*D*) which estimates the probability of two non-related isolates belonging to two different genomic types, according to the total number of types and isolates for each type (Hunter and Gaston, 1988; Simpson, 1949).

To further select the best degraders among representative strains from different origins (WWTP sample or four AEx with different carbon source) and isolation media (TSA or four selective media), a 10 sets intersection analysis was performed, using Venn's webtool (http://bioinformatics.psb.ugent.be/webtools/Venn/) for intersections count and UpSetR package (Conway *et al*., 2017) for *UpSet* plot design.

3.2.5 Profiling of microbial consortia

Community study was applied using third generation sequencing technology by *Oxford Nanopore Technologies*®, processed by BioISI Genomics Facility. Two taxonomically relevant ribosomal genes were chosen: 16S rRNA gene for prokaryotes profiling, and D1-D2 region of the 26S rRNA gene for fungi and protozoa.

Genomic DNA from AEx samples was obtained by modified GES protocol for DNA extraction. The primers pA and 1392R were used for 16S rRNA gene amplification (Lane, 1991), as previously described in Chapter 2, and library preparation was conducted by BioISI Genomics Facility, following an optimised protocol of Oxford Nanopore Technologies' using

SQK-RAB204 16S Barcoding Kit. For 26S rRNA gene, the Oxford Nanopore Technologies' *SQK-LSK109 Kit* was applied, using the primers NL-1and NL-2 for PCR amplification (O'Donnell, 1993; Guého *et al*., 1989). Adaptive evolution samples were sequenced after PCR amplification in a *GridION X5* platform using *MinKNOW* 18.08.2 software. The sequence data treatment was conducted in *Qiime2*, version 2019.1 (Bolyen *et al*., 2018), after *MinKNOW* 18.08.2 quality control classification of reads.

The taxonomic classification of OTUs was performed by a Naïve Bayes classifier, specifically defined from the reference database: the MiDAS taxonomic database for 16S (McIlroy *et al*., 2017), and the Ribosomal Database Project – RDP for 26S rRNA genes (Cole *et al*., 2014). To assess the diversity in genomic data, two estimators were calculated in *Qiime2*: the Simpson index (*D*) and the abundance-based coverage estimator (ACE). As previously described, the Simpson index (*D*) estimates the species diversity (Hunter and Gaston, 1988; Simpson, 1949). The ACE was developed to calculate the expected OTUs from the observed OTUs (Chao and Lee, 1992; Kim *et al.*, 2017). The package *Ampvis2*, developed by Andersen *et al*. (2018) for R computational language (R version 3.5.1), was used for Principal Component Analysis and heat-map construction from the genomic data.

3.3 Results and Discussion

3.3.1 Inoculum adaptation

The major concern when starting this AEx was microbial population viability and growth, given the low solubility of the carbon sources and, therefore its accessibility to the microorganisms. For a successful laboratory AEx, the microbial population must multiply to overcome the re-inoculation step and avoid a wash-out. Since the substrate interferes with indirect methods such as absorbance or dry weigh, the direct microscopic cell count was used to assess microbial growth in the first 60 AEx cycles (Figure 3.2).

As seen in Figure 3.2, overall growth was similar between different substrates, with an average of generations per cycle from 5.26 (with lubricant mineral oil) to 5.49 (with anthracene). These results show a viable and growing population from the WWTP sample was maintain in all AEx, continuously increasing the number of cells in each condition. The cell count showed relative stability over the initial 60 monitored cycles, as seen in Figure 3.2 (b), with cell count around 10^9 to 10^{10} cells in average. The substrates occupied different heterogeneity in phase partition along the water in the AEx. The anthracene and phenanthrene crystals accumulated on the side of the flasks, while the tristearin and lubricant mineral oil floated on the top of the water layer.

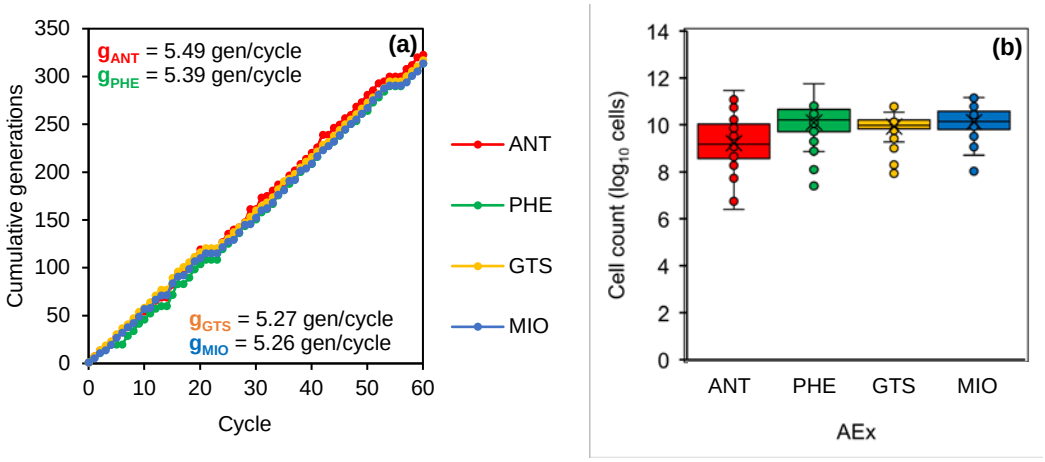


Figure 3.2 – Four adaptive evolution experiments followed by total cell count for 60 cycles. (**a**) Cumulative generation number along cycles; (**b**) Boxplots of total cell count per cycle in the different adaptive evolution experiments (AEx). *g* stands for the average number of generations obtained after each cycle, derived from the total cell count results. Each colour correlates to a different AEx: anthracene (ANT) in red, phenanthrene (PHE) in green, tristearin (GTS) in yellow and lubricant mineral oil (MIO) in dark blue.

This originated a structural liquid dispersion caused by continuous orbital shaking, with areas presenting high substrate presence (near the glass and top) and others with lower substrate distribution (in the centre and lower part of the flasks). This gradient might help the biodegradation process and cell multiplication, since microorganisms were not always in the same stress conditions.

It took around 30 cycles for visible and distinctive biomass development during the incubation cycle. In Figure 3.3 is visible the biomass growth in each AEx after 92 cycles, which was kept with the same pollutant as sole carbon source, from the original inoculum. Even though the initial community was the same, after approximately 500 generations and 92 "bottleneck" events, biomass differentiation was clear for each pollutant.

These differences can be explained by three hypothesis: microbial population divergency, with different degradative strains surpassing slower ones and presenting specific cellular phenotype (cell colour, accumulation of secondary metabolites), a convergence in the microbial population towards similar degradative populations in different AEx set-up which accumulated different secondary metabolites according to the carbon source available, or a mixture from both phenomena occurred. Visually, the PAH's proxies (anthracene and phenanthrene) both developed a dark brownish biomass, while for tristearin and lubricant oil, a mostly beige biomass could be observed. The visible biomass growth suggested a population evolution towards degradative microbial community, more adapted and resilient to the tested conditions. To my knowledge, this is the first report associating phenotypic change from a microbial community evolved from PAH or FOG pollutants.

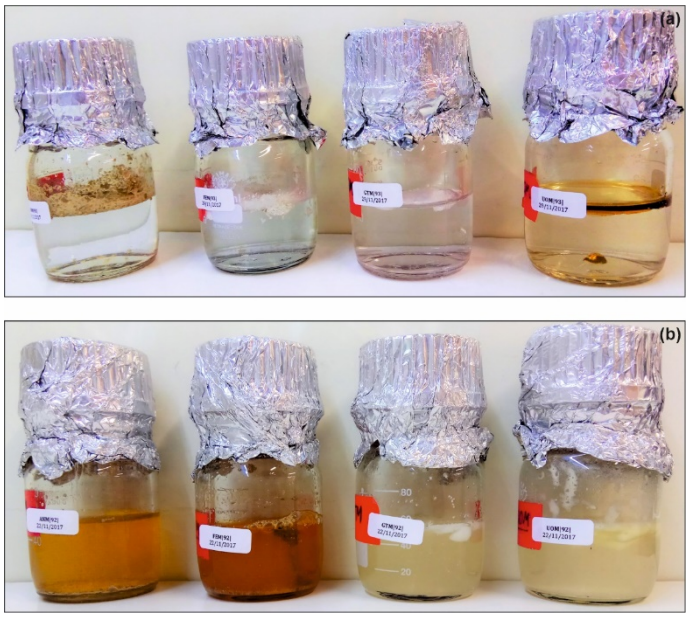


Figure 3.3 – Visible differences in the 92nd AEx cycle. **(a)**: the beginning of cycle, after the inoculation, with a clear aspect for each carbon source used; **(b)**: after 7 days incubation, with observable changes. A thick and visible biomass growth developed in each different AEx (from right to left): anthracene, phenanthrene, tristearin and lubricant oil.

The different AEx conditions caused visible biomass differentiation, which was also corroborated by isolation of biodegradative microorganisms and the genomic diversity study.

3.3.2 Isolation of microbial biodegraders

A total of 455 isolates were obtained, 30 yeasts and 425 bacteria and included in the BBC collection. These isolates were retrieved from a mixed natural inocula from the sludge of a WWTP (INI; 22 bacteria and 2 yeasts) and four different AEx, using pollutants as sole carbon source, namely anthracene (AEx-ANT; 89 bacteria and 6 yeasts), phenanthrene (AEx-PHE; 112 bacteria and 3 yeasts), tristearin (AEx-GTS; 85 bacteria and 4 yeasts) and lubricant mineral oil (AEx-MiO; 117 bacteria and 15 yeasts).

Considering their general characterization, isolates were clustered into five structural groups (I to V), namely Gram positive rods (Group I; mostly catalase positive and oxidase negative), Gram positive cocci (Group II; mixed catalase response and mostly oxidase negative), Gram negative rods (Group III; mostly catalase and oxidase positive), Gram negative cocci (Group IV; mostly catalase positive and oxidase negative) and yeasts (Group V). As seen in Figure 3.4, isolates obtained from the initial WWTP sample represent most of the collected Gram positive cocci (Group II). On the other hand, AEx-ANT (with anthracene) was responsible for the majority of Gram positive rods, while most Gram negative cocci isolates were retrieved from the AEx-GTS (with stearin).

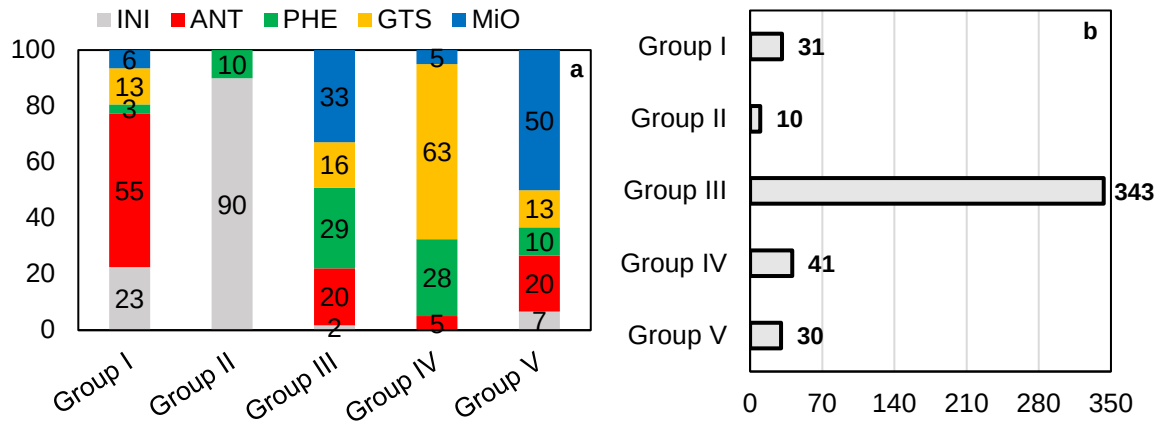


Figure 3.4 – Relative distribution of isolates over structural groups and divided by AEx experiment and total number of isolates in each group. **(a)** The structural groups were defined as: Group I for Gram positive rods; Group II for Gram positive cocci; Group III for Gram negative rods; Group IV for Gram negative cocci and Group V for yeasts. The isolates obtained from the initial inoculum are also represented (INI). (b) The total number of isolates distributed by the assigned structural group.

As for yeasts, half of the isolates were recovered from AEx-MIO (with lubricant oil). There was a clear dominance of Gram negative rod isolates, representing 75.4% (343/455). These results are in accordance with Kampfer and colleagues (1996), who analysed the microbiological composition of WWTP samples and found a dominance of Gram negative bacteria, mostly Proteobacteria, in both culture-dependent and -independent approaches, pointing for their functional relevance. However, Forster *et al*. (2002) found only <10% of Gram positive bacteria in WWTP samples, but representing the most relevant group for the biological activity of the flocs. The reports show the variability and heterogeneity found in activated sludge of WWTP, regarding bacterial diversity and the impact on the biological processes.

The distribution of isolates by structural groups and isolation source (natural consortium and the five AEx) showed significant association between groups (chi-square test; $p<0.0001$). While Gram positive rods were significantly associated to AEx-ANT, the Gram negative rods were mostly related to AEx-PHE. For the isolates from AEx with FOG proxies: tristearin (AEx-GTS) favoured the isolation of Gram negative coccus, while mineral oil (AEx-MIO) promoted both Gram negative rod bacteria and yeasts (chi-square test; $p<0.005$). These results support the study of the individual impact of these recalcitrant pollutants as the evolution force during the AEx. To assess the microbial evolution along time, all AEx were screened at different cycles, to isolate biodegraders in the selective media and in TSA (data summarized in Figure 3.5).

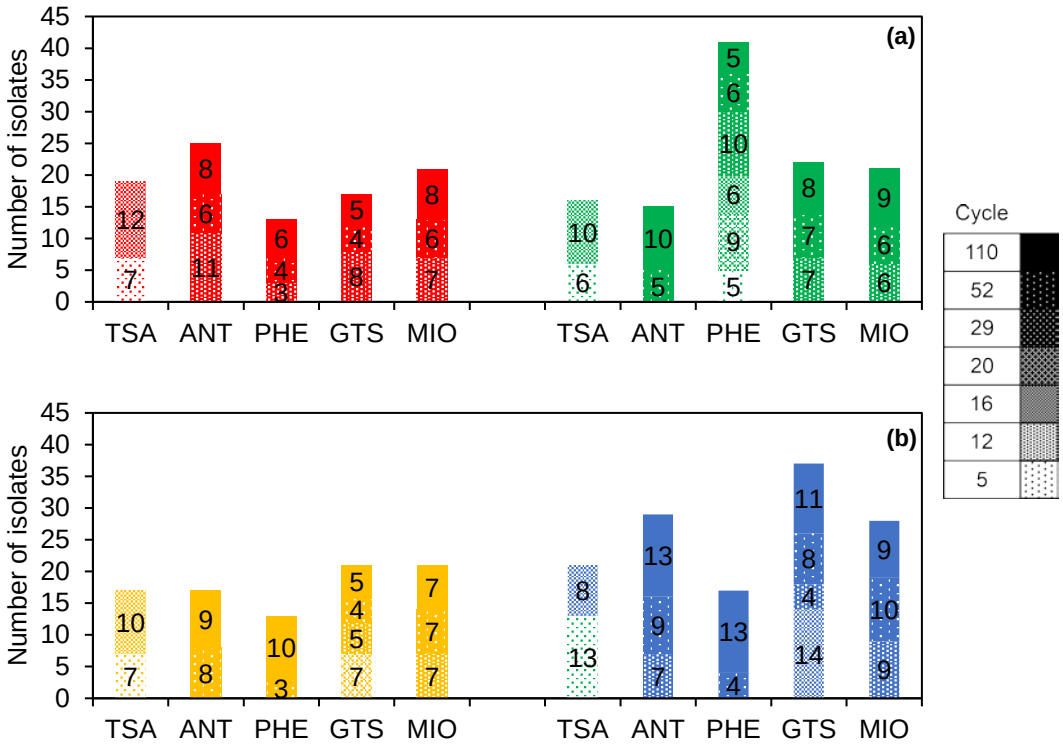


Figure 3.5 – Number of isolates obtained in each AEx, according to isolation medium and time cycle. Bar colour identifies the different AEx depending on substrate: anthracene in red and phenanthrene in green (**a**); tristearin in yellow and lubricant mineral oil in blue (**b**). The column shade indicates the number of AEx cycles before isolation, from 5 (spaced dots) to 110 cycles (full coloured), as depicted in the greyscale label on the right.

Isolates could be sorted by their AEx origin, cycle of isolation and growth media (four AEx, seven isolation cycles and five distinct isolation media). The isolation results according to isolation media showed an even distribution, with 97 isolates from general growth media TSA, and 86 from anthracene, 84 from phenanthrene, 97 from tristearin and 91 from lubricant mineral oil selective growth media. On the other hand, the results according to the isolation cycle showed a variable distribution, as seen in Table 3.2. Initially, it was expected to obtain few biodegraders from the initial WWTP sample, given the usual presence of hydrophobic pollutants (FOG and hydrocarbons) in WWTP. However, it took 29 cycles of AEx to successfully isolate microorganisms in the selective media. This was most likely related to the evolution of more adapt and resilient strains, able to grow in the selective solid media. Considering the PAHs, phenanthrene selective medium was the first substrate to present visible colonies (at the 5th cycle), while with anthracene it took 29 cycles to obtain the first isolates. Both substrates are PAH, with three aromatic rings, presenting similar chemical features. However, due to the angular difference in phenanthrene, both substrates present specific known metabolic pathways, starting from specific dioxygenase activity. Several bacterial strains have shown biodegradation pathways for both substrates, as *Mycobacterium*

sp. PYR-1. It was reported to use both anthracene and phenanthrene through similar metabolic pathways, differing in the initial dioxygenase ring fission site and subsequent path to phthalic acid metabolism (Moody *et al*., 2001). Other strains, as *Burkholderia* sp. C3 and *Paeniglutamicibacter sulfureus* RJK4 (formerly *Arthrobacter sulfureus*; Busse, 2016), have shown ability to degrade phenanthrene with a similar pathway, without a reported capacity for another PAH degradation (Samanta *et al*, 1999; Seo *et al*., 2006; Seo *et al*, 2009). Nonetheless, these results could suggest the evolution of more specialized strains, with reinforced PAH metabolism activation, given the continuous presence of specific pollutant as sole carbon source.

Table 3.2 – Number of isolates according to AEx isolation cycle and the selective medium.

AEx cycle	***Selective Medium Substrate***				
	TSA	**ANT**	**PHE**	**GTS**	**MIO**
0	24	0	0	0	0
5	*na**	0	5	0	0
12	33	0	0	0	0
16	0	0	9	7	0
20	40	0	6	14	0
29	*na**	18	13	24	29
52	*na**	28	17	23	29
110	*na**	40	34	29	33

** not assessed*

The above data showed the overall success in the AEx, considering the main goal to isolate biodegraders for future bioremediation product development. It allowed to obtain potentially improved degradative isolates by evolving a natural microbial population from a man-made polluted site. Also, the structured media proved a challenge to work with, when compared to routinely used soluble carbon sources, such as glucose or sucrose. Given the very low solubility in water of anthracene and phenanthrene, respectively 0.000044 and 0.0012 $g.kg^{-1}$ H_2O (Haynes *et al*., 2017), and also tristearin and lubricant oil, these substrates retain their structure, interfering with common measurement approaches, such as optical density. This forced the application of methods such as direct cell count and selective growth media to retrieve degradative strains. Other authors have successfully used similar approaches, applying most probable number with selective media to enumerate hydrocarbon degrading microorganisms in seawater and contaminated soils (Coulon *et al*., 2005; Kasai *et al*., 2002) to assess biodegradation capacity of environmental microbiome.

3.3.3 Genomic diversity

To assess the genomic diversity of the BBC collection, three single-primer PCR fingerprintings were used as previously described. As discussed in *Chapter II – Genomic diversity and biodegradation ability of isolates*, the discriminatory level for the method was determined at 90% similarity (data not shown). Isolates with genomic similarity of at least 90%, presenting congruent phenotypical group and retrieved from similar inocula (same AEx), were considered indistinguishable and were clustered into a single representative, the isolate from the newest cycle, potentially corresponding to the most evolved isolate. The genomic profile allowed to establish congruent clusters of isolates, leading to representative strain allocation. From this analysis, 25% (115/455) of isolates were considered distinguishable, turning into representative strains (Figure 3.6), while on the other end, two representative strains aggregate 33 indistinguishable isolates each. The clustering led to a 60% reduction of the initial 455 isolates to 182 representative strains, with combined phenotypic characterization and experimental data from each of the indistinguishable isolate. This approach allowed to see the overlapping traits from the isolates and assess multiple traits from the obtained isolates. As showed in Figure 3.7, by combining the data, each representative strain could be related to different AEx, various cycles and multiple selective media.

In accordance with the isolation results, Gram negative rods (Group III) were also the most abundant set (139 representatives), followed by Gram negative cocci (Group IV) and Gram positive rods (Group I), with 16 and 15 representatives, respectively, and 6 representatives for both Gram positive cocci (Group II) and yeasts (Group V).

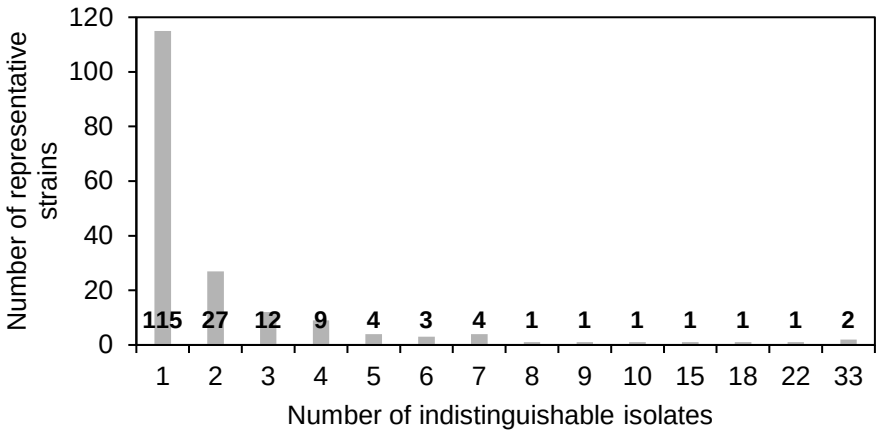


Figure 3.6 – Number of representative strains according to the number of embodied indistinguishable isolates.

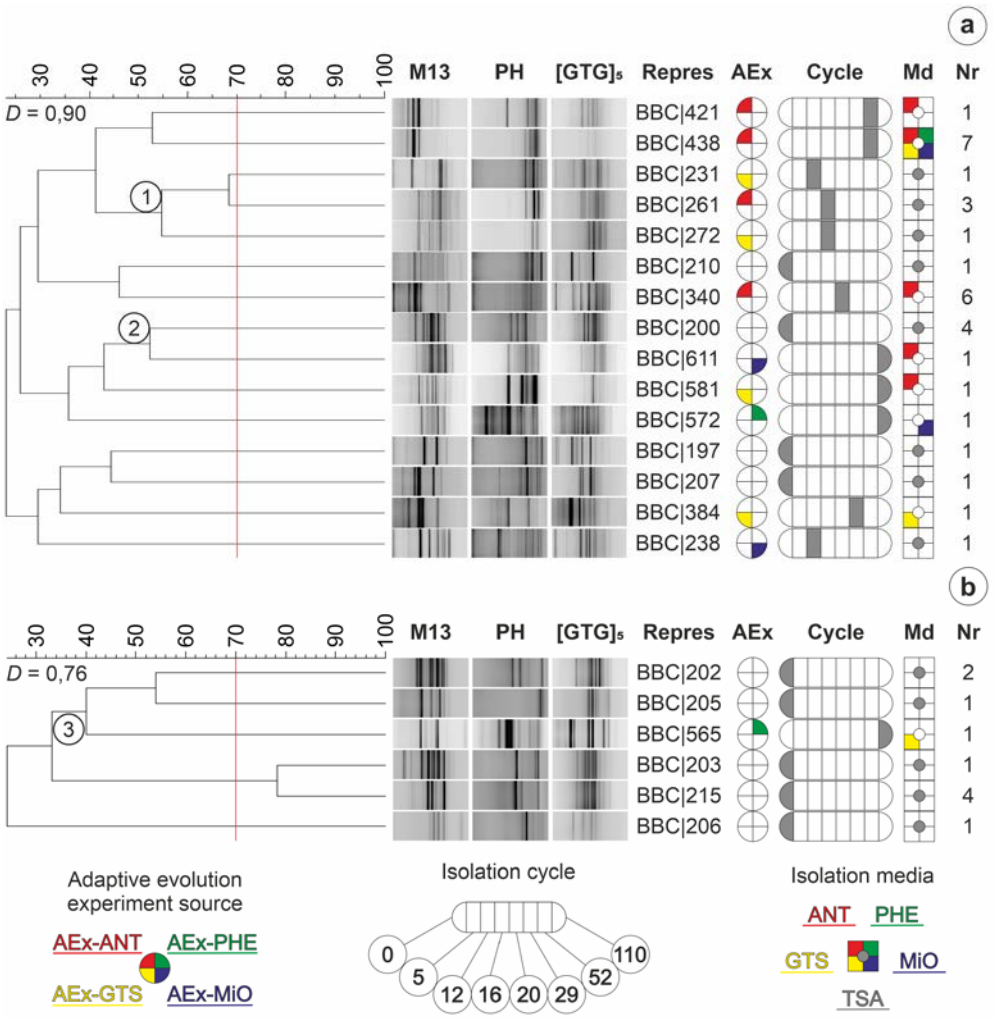


Figure 3.7 – Genomic relatedness of representative strains and phenotypical feature (AEx source, isolation cycle, and isolation medium) divided by structural group and AEx in the case of Group III. **(a)** Group I: Gram positive rods; **(b)** Group II: Gram positive cocci; **(c)** Gram negative rods (Group III) from AEx-ANT; **(d)** Group III from AEx-PHE; **(e)** Group III from AEx-GTS; **(f)** Group III from AEx-MIO; **(g)** Group IV: Gram negative cocci; **(h)** Group V: yeasts. In each dendrogram, the representative strains with the same structural group, obtained from the initial WWTP sample, were also included. The dendrogram was based on PCR-fingerprinting with primers csM13, PH and $[GTG]_5$, using Pearson correlation coefficient as similarity measure and UPGMA as clustering method. The threshold line for assessment of Simpson's diversity index is represented at 70% similarity, and in the top left corner the calculated value considering the number of isolates obtained (*D*). Representative strain reference number (**Repres**), adaptive evolution experiment (**AEx**), cycle of isolation (**Cycle**), selective medium used for isolation (**Md**) and the number of isolates it represents (**Nr**) is also displayed (BBC corresponds to bacteria and YBC to yeasts included in the *BioTask Bioremediation Collection*). Numbered clusters indicate genomically similar representative strains from different AEx experiments and cycles (1 to 24). For colour codes see the bottom of each part of the figure.

The Gram negative rods include known biodegraders for PAH, such as *Pseudomonas*, *Acinetobacter*, *Burkholderia*, or other genera, which could explain the highest number of representative strains (Seo *et al*., 2006; Seo *et al*, 2009). There are also reports of genera of Gram positive bacteria described as biodegraders for PAH, as *Arthrobacter*, *Mycobacterium*, or *Paenibacillus* (Samanta *et al*, 1999; Moody *et al*., 2001; Bisht *et al*., 2015; Dudhagara and Dave, 2018), although researchers have more frequently reported *Proteobacteria* as having a major role in PAH bioremediation processes.

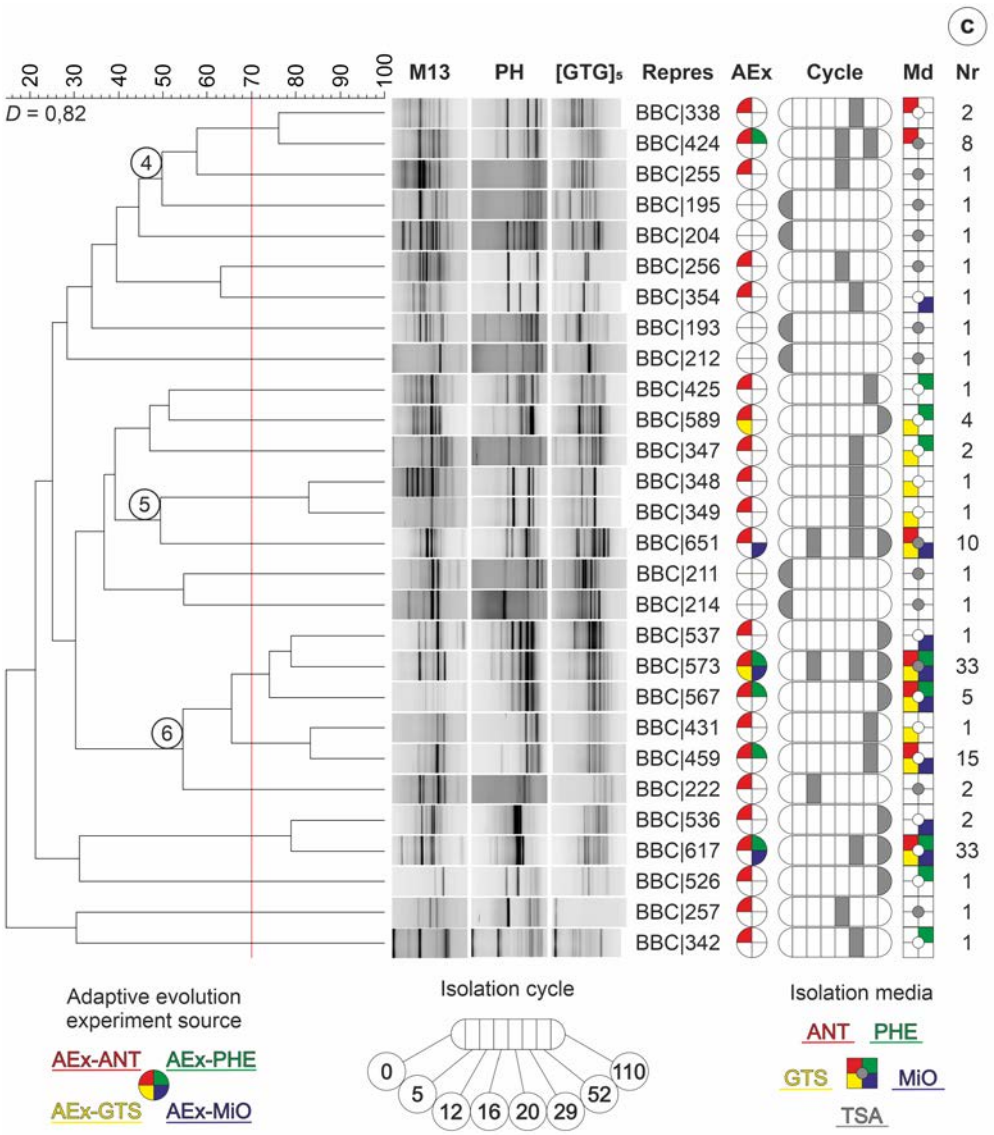
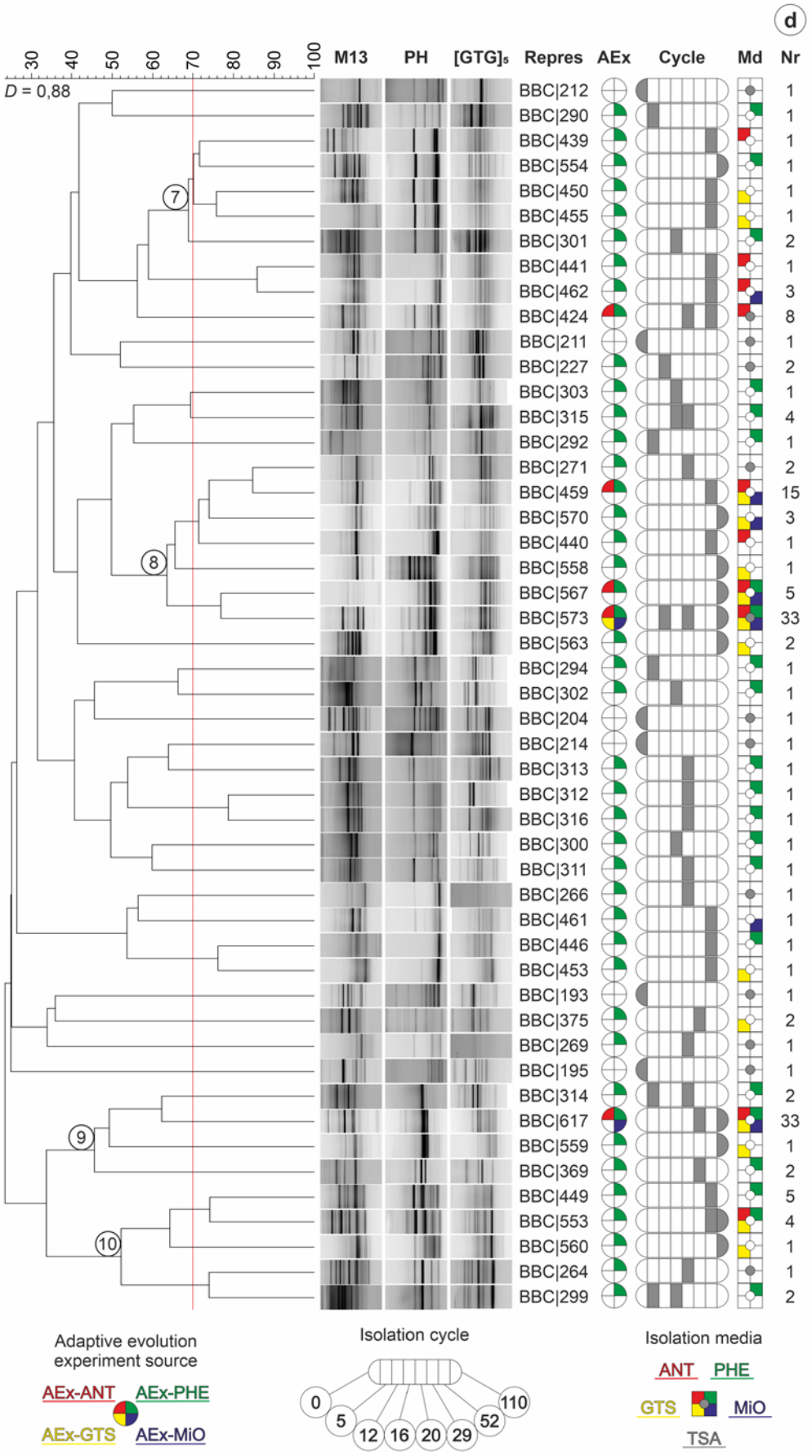


Figure 3.7 (continued)

In Figure 3.7, a summary of all data is shown for the selected representative strains, displaying the high level of diversity retrieved from the AEx. Concerning overall diversity, Group I (Figure 3.7a) showed the highest diversity level with 90%, followed by Group III (Figure 3.7c, d, e and f) with values from 82% to 88%, and the smaller groups (Group II, IV and V), with diversity values of 76%, 70% and 51%, respectively. Natural samples from highly contaminated sites as WWTP have reportedly been associated with high diversity indexes, from both culture dependent and independent methods (Cydzik-Kwiatkowska and Zielińska, 2016; Wu *et al.*, 2019).



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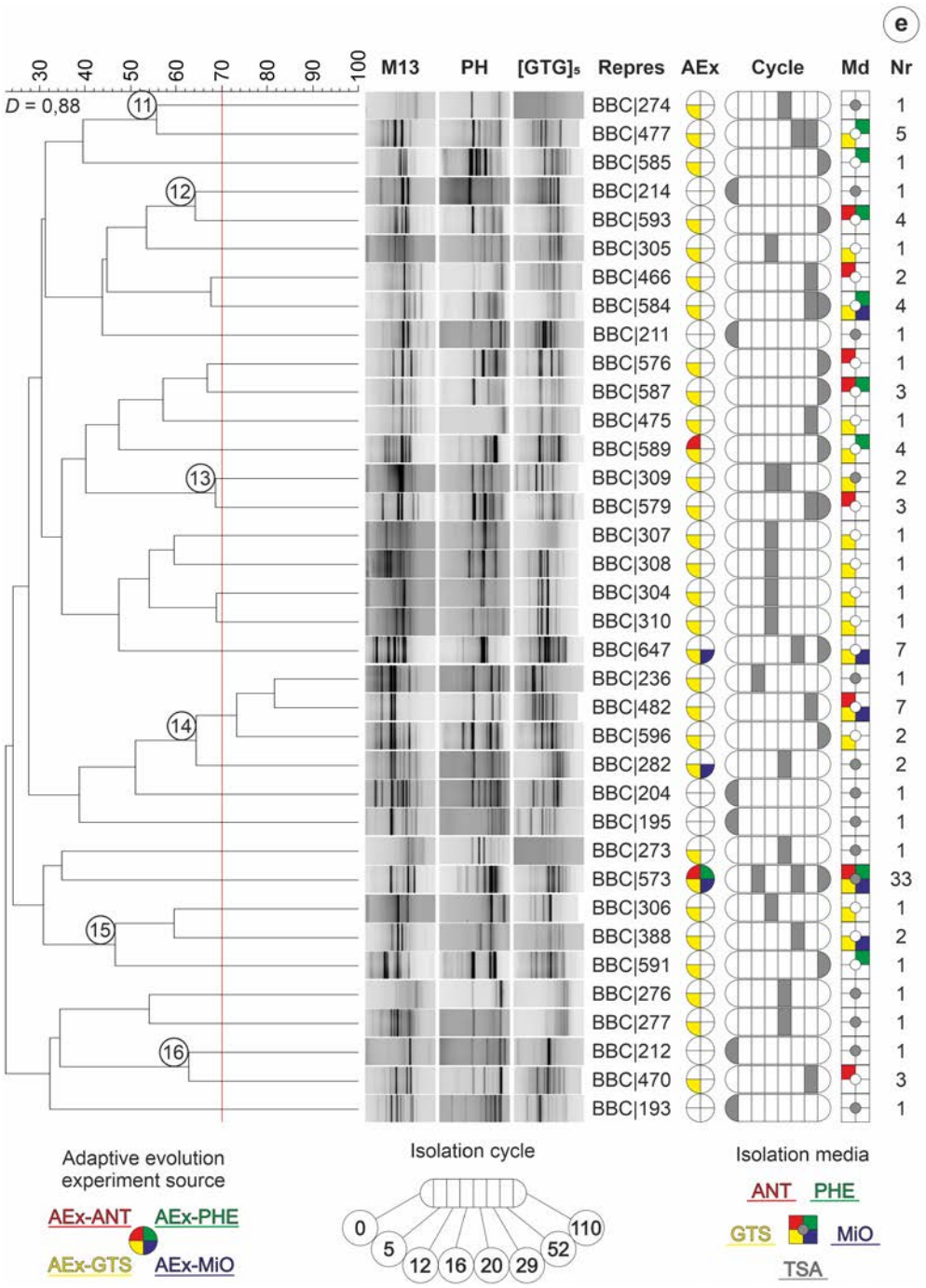


Figure 3.7 (continued)

However, AEx results show a clear tendency towards gram negative rods (Group III), in both number of isolates, representative strains, and diversity. Group III was the most represented group, in all AEx experiments, which could be explained by similar metabolic pathways involved in both PAH, aliphatic hydrocarbons and triglycerides degradation, as discussed in Chapter I. The common origin for WWTP sample used in all AEx supports the isolation of same representative strains in different conditions.

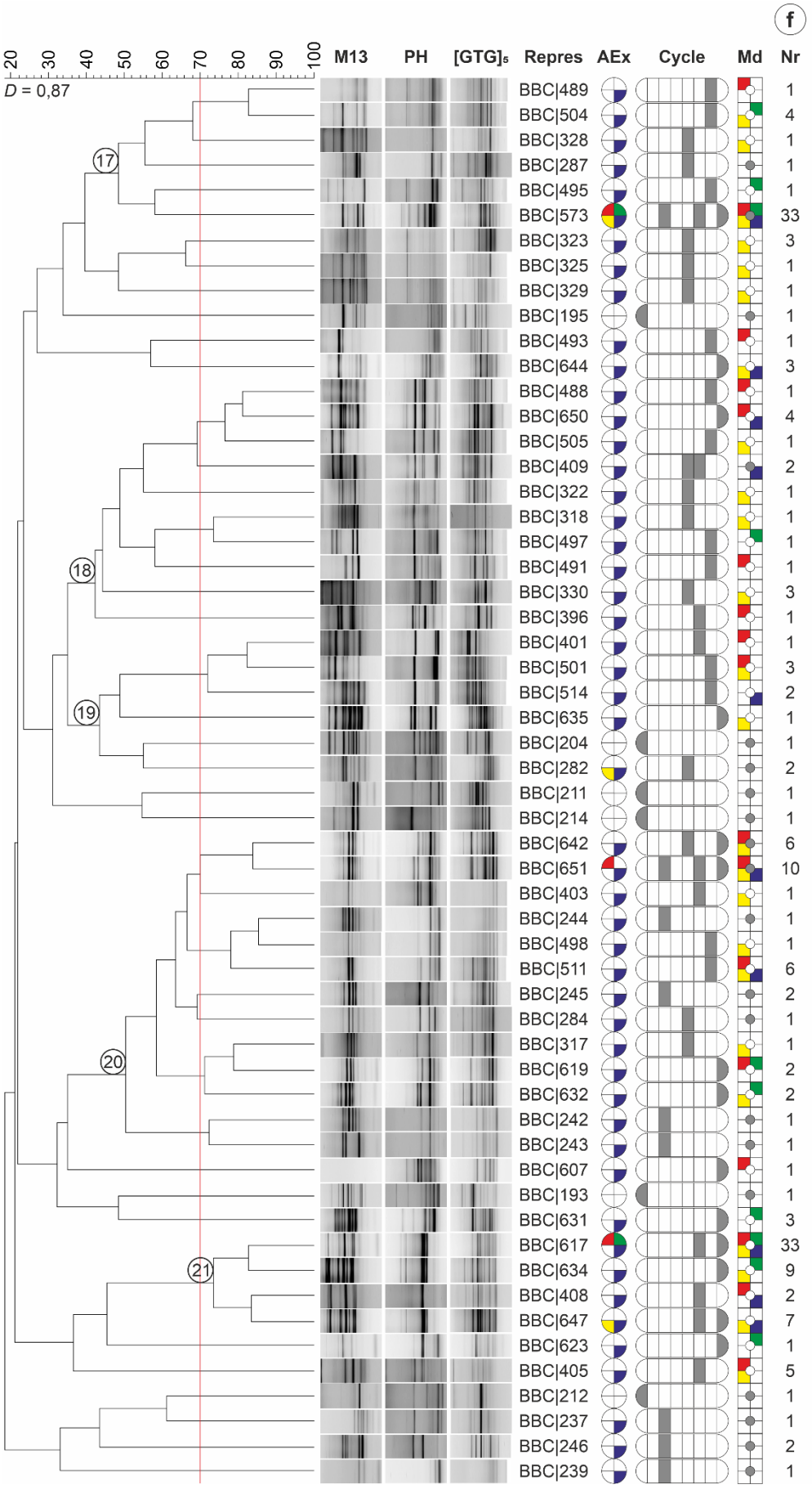


Figure 3.7 (continued)

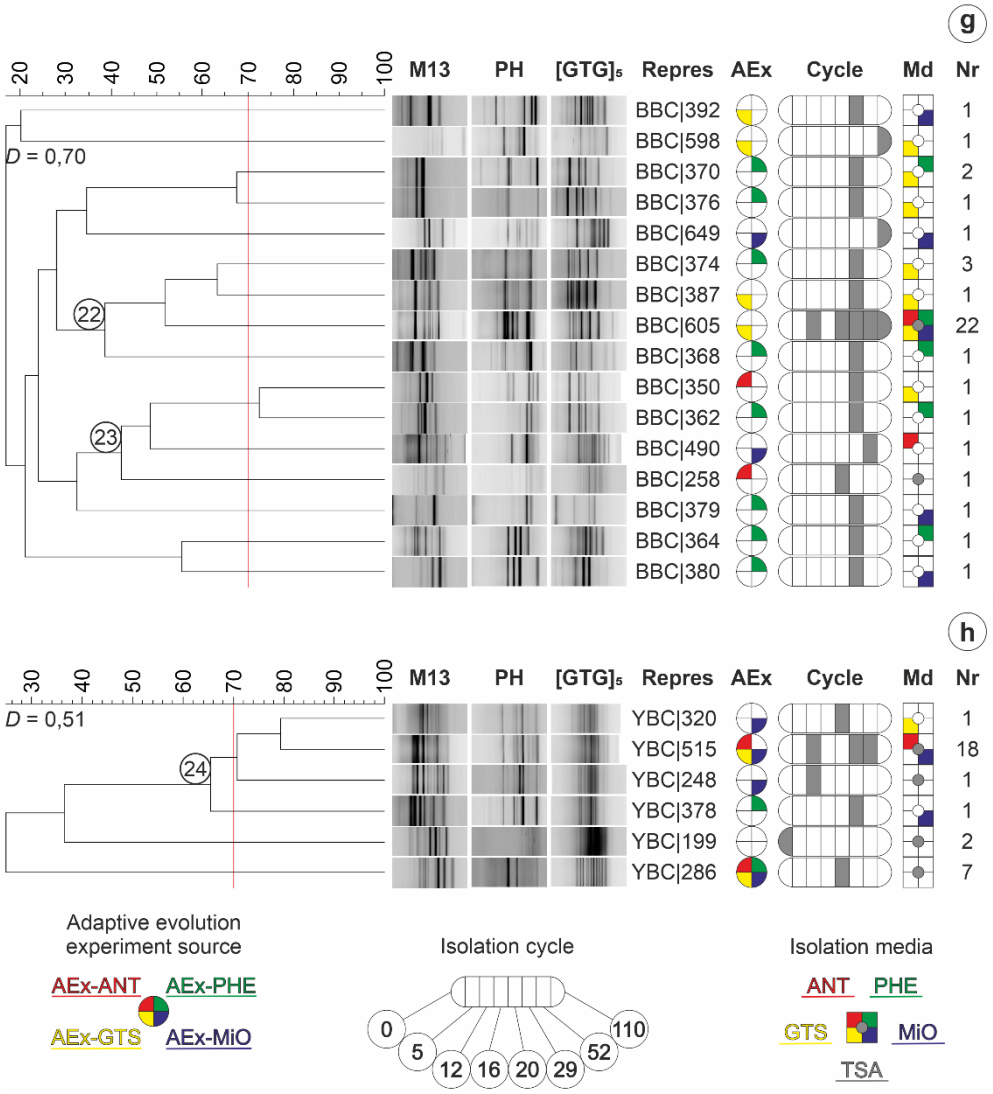


Figure 3.7 (continued)

Although degradation pathways are usually induced by substrate, as the *alk* genes metabolic pathway in *Pseudomonas*, which requires the presence of alkanes to actively promote the necessary metabolic machinery synthesis (Nzila, 2018), it seems that many degradative representatives presented a broad-spectrum enzymatic machinery. And so, even though each AEx contained different pollutants as sole carbon source, several strains were able to thrive in multiple AEx. A fine discriminatory tool, as the 3 independent PCR-fingerprinting genomic profiles, unveiled different relatedness levels from low similarity clusters along the established groups. The numbered clusters along Figure 3.7 indicate representative strains obtained from different AEx experiments and/or cycles, pinpointing interesting relatedness between them. Regarding clusters 7, 10, 11, 15 and 18, all contained representative strains obtained from the same AEx and different cycles. The strains obtained from later AEx cycles could represent derived strains, caused by the stress-induced environment, forcing the

evolution for more adapted strains. As an example, cluster 15 shows three strains from cycle 16 to 110, all from AEx with tristearin (GTS) and from different selective media. Interestingly, isolates from later cycles were more frequently found in different AEx assays or in multiple selective substrates, which suggests the stress induced by the AEx forced the selection of degradative populations, presenting the necessary active metabolic pathways for pollutants biodegradation.

As discussed, the AEx adaptation may occur by accumulation of small genetic variations which would affect the genomic fingerprinting used, since three different methods were applied. Clusters 2, 3, 4, 9, 10, 12, 16, 19 pinpoint genomically related representative strains from quite different cycles. The most drastic example was cluster 2, with isolate BBC|200, from the initial WWTP sample and BBC|611, from the 110 cycle of the AEx with mineral lubricant oil, presenting quite small differences in the genomic profiles. Taking into account the numbered clusters with strains from multiple AEx and cycles, the clusters 1, 5, 7, 8, 11, 13, 14, 15, 18, 22, 23 and 24 serve as examples. In the first, strains from the 12^{th} and 16^{th} cycle, both AEx with anthracene and tristearin, and all isolated in TSA, show small differences.

Furthermore, cluster 4 indicates a proximity between representative strains from the initial sampling (BBC|195) and from AEx with anthracene and phenanthrene, from much later cycles (52^{nd}, 29^{th} and 20^{th}), from selective growth medium with anthracene. It is worth mentioning that the only yeast cluster, the 24^{th} cluster, represents isolates from all AEx substrates, found in several cycles (12^{th}, 20^{th}, 29^{th} and 52^{nd}), and in all selective media, except with phenanthrene. This proves the continuous presence of a close related yeast population over all adaptive evolution experiments.

From the isolation results, the yeasts comprised a small population in the initial inocula, even though presenting relevant metabolic features, as proven by the selective media isolation. For last, the clusters 6, 17 and 21 represent the most metabolically diverse representative strains, from all AEx, several cycles and multiple selective media. As seen in cluster 6, representative strains were encountered in all AEx, from the 12^{th}, 29^{th}, 52^{nd} and 110^{th} cycles and all selective media, with closely related genomic profiles. These clusters include quite plastic metabolic strains, capable to grow on the tested pollutants. The AEx seem to have broaden the metabolic capacity of these strains, since the isolates from later cycles (52^{nd} and 110^{th}) were isolated from AEx with different substrates and the earlier strains, from the 12^{th} cycle, were only obtained from AEx-ANT.

Given the genomic relatedness displayed in Figure 3.7, several clusters present evidence of evolution under a carbon limiting, stress induced experiment. Either by genetic mutation acquired over time, or insertion/deletions events of genetic material or small genetic elements trade (horizontal gene transfer), this laboratory adaptive evolution forced strain improvement. The bottleneck approach, which caused the wash-out of slow growing strains, promoted the isolation of adapted, resilient strains, capable of using the available pollutant as sole carbon source and prevail in a competitive environment.

3.3.4 Consortium dynamics along adaptive evolution

A 3rd generation DNA sequencing approach, for high-throughput genomic analysis, allowed to follow overall evolution of the microbial populations during the AEx. The culture-independent approach presents a wider response range towards microbiological diversity, without the technical constraints given by a culture dependent one. A main concern for such methodologies resides in reference curated databases with appropriate target association, since a database with full known diversity would compromise data analysis with unbearable time-consuming assignment. For this reason, the MiDAS project (McIlroy *et al*., 2017) presents a manually curated reference database specifically designed for wastewater microbiome, targeting the 16S rRNA gene sequence, while the Ribosomal Database Project (Cole *et al*., 2014) offers a general reference database for 26S rRNA gene sequencing data, since no specific database focusing on wastewater microbiome exists for this genetic target. Both reference datasets have been successfully used with similar data, to assess diversity among different WWTP or to cope seasonal impacts over microbial populations (McIlroy *et al*., 2015; Zhang *et al*., 2018). In the present work, applying the NanoPore® technology allowed to analyse almost 3.9 million reads, from two targeted rRNA genes (16S and 26S) in nine different samples, as reported in Table 3.3. This methodology has been thoroughly compared and validated, with sequencing quality similar to Sanger technology, as discussed by Wurzbacher and colleagues (2019). In terms of number of reads, all samples obtained over 89,000 quality reads, with exception to 16S rRNA gene sequencing from the natural WWTP sample, used to inoculate the AEx. This could be a result of low content of genomic material from bacteria to start with, the low number of cells, and the presence of small sediments and contaminants, could have hampered the extraction and amplification process. Given the small sample size (110 reads), the diversity and richness estimators most probably do not represent the actual original sample. Nonetheless, for the remainder samples and both genetic targets, there was no adverse effect, obtaining a high number of quality reads.

Table 3.3 – Microbial profiling indicators from metagenomic data. The number of reads corresponds to the reads which passed the quality control from Nanopore software. The Observed OTUs corresponds to que number of different taxa obtained from taxonomic assignment. Simpson index and ACE calculated with *AmpVis2* R package. Results from all AEx, with cycle number in square brackets (52nd and 110th cycle): anthracene (ANT); phenanthrene (PHE); stearin (GTS); lubricant mineral oil (MIO). WWTP sample refers to the initial inoculum. Two different rRNA genes were amplified by PCR: 16S, for bacteria; and 26S, for fungi.

	Number of Reads		***Observed OTUs***		***Diversity estimator***		***Richness estimator***	
					Simpson index		**ACE**	
rRNA target	*16S*	*26S*	*16S*	*26S*	*16S*	*26S*	*16S*	*26S*
WWTP sample	110	86,680	26	38	0.78	0.67	54	55
ANT [52]	40,522	118,429	82	24	0.83	0.83	120	26
ANT [110]	1,280,093	89,825	61	39	0.81	0.78	70	43
PHE [52]	589,152	195,738	91	45	0.83	0.81	131	53
PHE [110]	751,367	102,329	49	41	0.69	0.88	55	45
GTS [52]	1,230,950	162,569	74	31	0.60	0.80	83	37
GTS [110]	528,801	147,592	69	35	0.78	0.83	88	39
MIO [52]	106,756	196,331	41	17	0.85	0.76	45	19
MIO [110]	432,311	222,947	52	38	0.83	0.85	61	43

Regarding the number of observed Operational Taxonomic Units (OTU), the lowest value was 17, observed in the AEx-MIO, 52nd cycle for fungi, and the highest was 91, for AEx-PHE, 52nd cycle for bacteria, which are in the same range as the ones used to define the MiDAS database, when analysing several Danish WWTP microbiomes (McIlroy *et al*., 2017). However, other reports indicate a much higher diversity, with 1,932 OTU in effluent from WWTP (Ye and Zhang, 2013) and even <3.000 OTUs (McLellan *et al*., 2010). These disparities show the diversity observed in different WWTP and could result from different extraction methods, sequence technologies and data analysis algorithm. The diversity indexes were similar to values from the genomic differentiation of isolates previously discussed, which was not expected. Most high-throughput genomic approaches usually reach much higher diversity and richness values, above 95% and 1,000, respectively (McLellan *et al*., 2010; Hu *et al*., 2012; Ye and Zhang, 2013).

The richness estimator changes indicate an overall reducing tendency from the 52nd cycle to the 110th cycle in the AEx with PAH for bacteria and fungi. However, in the AEx with FOG, the ACE show an increase in global richness in the same cycles. It would be expected an overall decrease in the expected number of OTUs along AEx, given the continuous bottleneck

effect. However, the adaptative stress caused by the FOG seemed to have increase this tendency, while for PAH there was an overall decrease, with exception for the fungi population in AEx-ANT. Moreover, the number of observed OTUs indicate a higher diversity of bacteria rather than fungi in overall AEx samples, congruent with the previous discussed isolation results.

In Figure 3.8 are represented the top 15 most frequent OTU found in the genomic analysis in a heat-map. In the case of the 16S rRNA gene (Figure 3.8a), a considerable fraction of the sequences (average 21%) could not be assigned to any taxa, identified as *k_Unassigned*, or was just associated at the kingdom level (*k_Bacteria*, average 30%). These could correspond to small underrepresented groups in the MiDAS database, which would not be expected given the specificity of the database used, specifically designed for WWTP communities. They could also be a result from small genetic mismatch, which fail to be assigned at least at phylum level or present too many mismatches to be assigned to a specific taxonomic group. Similar values were described for Illumina technology, by Hu and colleagues (2012), reaching over 30% of unclassified bacteria.

The most predominant phylum was Proteobacteria in all samples, ranging from 23.6% to 46.6%, distributed along five classes: Alfa-, Beta-, Gamma- and Deltaproteobacteria. All AEx samples presented higher presence of Proteobacteria, suggesting an increase in this highly diverse phylum. While the maximum value for the Proteobacteria phylum was obtain in the 52nd cycle, the results show different distribution between classes along each AEx. For AEx-ANT, there was an initial increase in Gammaproteobacteria (39.2%), followed by a shift towards Beta- and Alphaproteobacteria (17.5% and 18.3%, respectively). On the case of phenanthrene, the Alphaproteobacteria were initially dominant (25.6%) and were supplanted by the Betaproteobacteria (41.3%) at the 110th cycle. As for FOG pollutants, both promoted the presence of Gammaproteobacteria after 52 cycle, with 36% and 36.9%, but with divergent evolution. In the last cycle, while for lubricant oil (MIO), the there was only a small increase in the dominance of the Gammaproteobacteria, the tristearin forced a shift along Gammaproteobacteria, Betaproteobacteria and Alphaproteobacteria (14.7%, 6.7% and 14%).

The second most represented phylum was Parcubacteria, which was only surpassed by the Bacteroidetes phylum (4.9%) in the 110th cycle of the AEx-GTM. The results reinforce the importance of the phylum Proteobacteria at a community level, in wastewater microbiome. The Parcubacteria, a candidate phylum, has been associated to anoxic conditions and fermentative metabolism, apparently capable of degrading complex carbon sources such as cellulose and chitin (Nelson and Stegen, 2015).

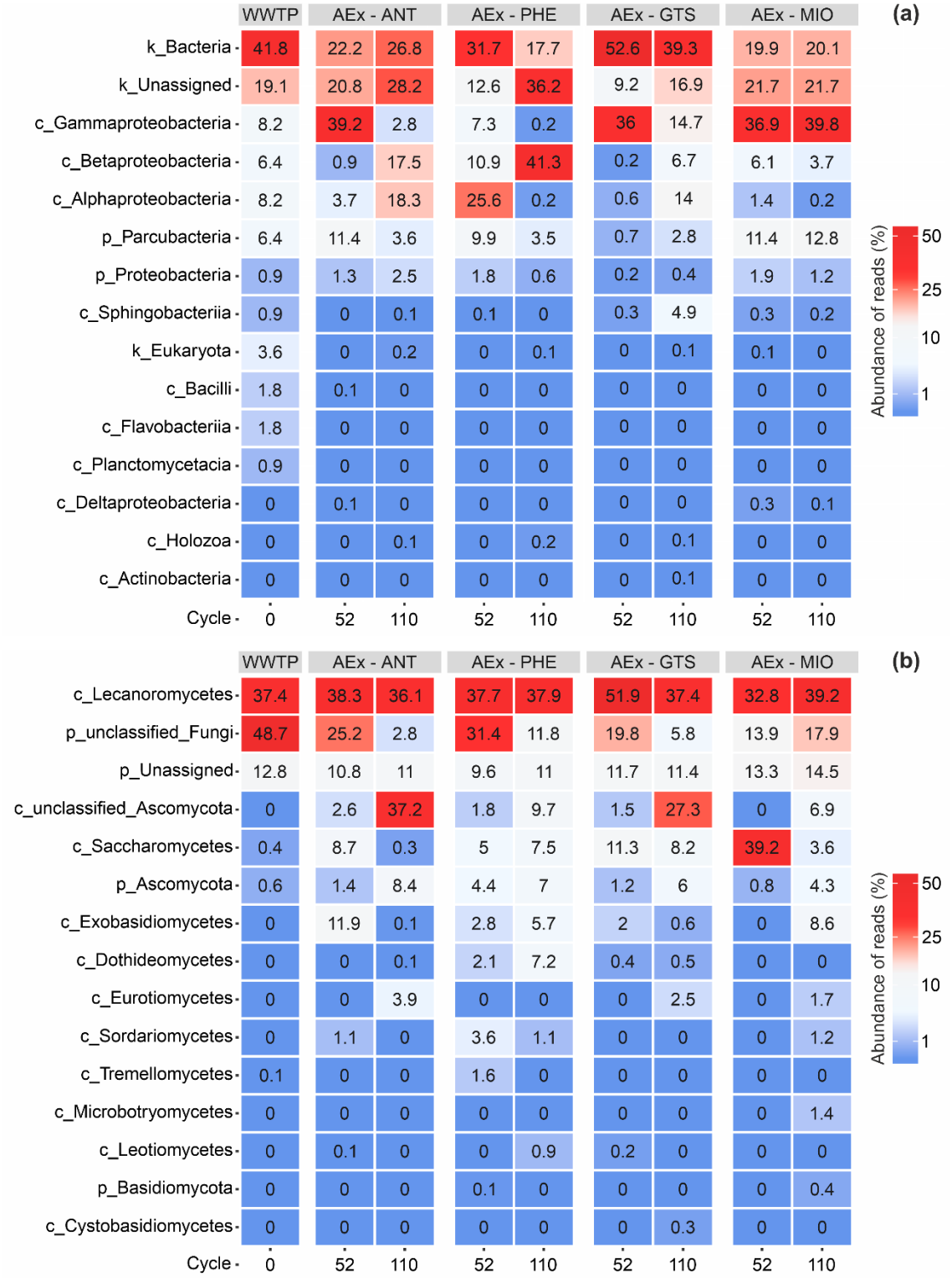


Figure 3.8 – Heatmap of taxonomic classification from gene sequencing results. Relative abundance of the 15 most abundant taxa, for 16S rRNA (**a**) and for 26S rRNA (**b**). The reads were classified using a specifically design Naïve Bayes classifier with *Qiime2* software, with a confidence threshold of 80%. The colour represents the relative read abundance from 0 to 50%, as depicted in label. Results are paired by AEx (anthracene, AEx-ANT; phenanthrene, AEx-PHE; tristearin, AEx-GTS; lubricant mineral oil, AEx-MIO) and cycle number in bottom (52nd and 110th). The WWTP refers to the natural microbiome used to start the AEx (cycle 0).

The results show an increase in the incidence of this group during the AEx, with exception for tristearin (AEx-GTS) were a small reduction was observed. In the case the triglyceride, the second most represented group at the final cycle was the Sphingobacteriia class, from the phylum Bacteroidetes (4.9%). It has been shown a major prevalence of such diverse groups (Alpha-, Beta- and Gammaproteobacteria) in WWTP, with smaller but consistent contribution from other, as Firmicutes phylum. In this study, only in the initial natural sample was found a relevant community of class Bacilli, which disappeared during the adaptive process. This could represent slow growing groups, which were washed-out during the process, or simply could not adapt to the conditions of adaptive experiment.

Noteworthy, the presence of Eukaryota domain OTU, associated to flagellates and ciliates which were mostly observed on the initial inocula sample (3.6%). The low abundance of Eukaryota reads found in the remaining samples suggest a reduction of these communities in all AEx. In the case of the fungi community, shown in Figure 3.8b, the taxonomic changes along AEx were more pronounced. Overall, there were less proportion of unclassified reads, representing an average of 41% of OTU for each sample. The most frequent taxon in all samples was Lecanoromycetes class, from Ascomycota phylum, one of the largest class of lichenized Fungi, and one of the most species-rich classes in the Fungi kingdom (Miadlikowska *et al*., 2014). This class has been previously reported in several industrial WWTP in low abundance (Selvarajan *et al*., 2019), most likely transported by water and air from the environment surrounding the infrastructure. The samples from AEx-ANT show an increase in Lecanoromycetes (38.3%), Sacharomycetes (8.7%) and Exobasiodiomycetes (11.9%) classes at the middle of the assay, culminating in a majority of unclassified Ascomycota (37.2%), followed by Lecanoromycetes class (36.1%). These results indicate a high percentage of unknown Ascomycota contributed to the population evolution with anthracene as sole carbon source. For AEx with phenanthrene, Lecanoromycetes class covered 37.7% and 37.9% in both cycles, while other classes increased their presence, namely Saccharomycetes (7.5%), Exobasiodiomycetes (5.7%), and Dothideomycetes (7.2%). In the case of tristearin, the community changes were seen by the increase in Saccharomycetes (8.2%), Exobasiodiomycetes (2%), Dothideomycetes (0.5%), and Eurotiomycetes (2.5%) classes, and the consolidation of unclassified Ascomycota (27.3%). The lubricant mineral oil (AEx-MIO) originated the most dramatic community shift, favouring Saccharomycetes class (39.2%) by the 52nd cycle, and culminating in a much more diverse profile, with the previously referred fungi classes represented in small numbers and a majority of Lecanoromycetes class. The 3rd generation sequencing allowed to assess changes occurring

within the initial WWTP inoculum community throughout each AEx without the limitations of culture-dependent techniques. In the case of bacteria, the results show partial consistence with the isolation efforts, considering the main groups distribution and the high prevalence of gram-negative rods in the BBC.

Concerning the 26S rRNA results, the disparity between culture-dependent methods was more noticeable. Several microbial groups were genomically detected, and the most frequent one (Lecanoromycetes class) was not isolated. In fact, without the genomic approach one might think there was a low abundancy and diversity among Fungi during the AEx, when in fact, this group clearly had been evolving from the initial consortium.

Given the multiple variables, samples and conditions to explore, a Principal Component Analysis (PCA) was applied as an integrative approach to study the impact of AEx over the initial consortium community. The PCA retained at least 70% of the original variance of data, providing a fair representation of the obtained data for both bacteria and fungi (Figure 3.9 and Figure 3.10). For bacteria, the AEx samples were distanced from the initial consortium specially by the PC1 for the 52nd cycle, and by PC2 for the 110th cycle samples (Figure 3.9a). In the 52nd cycle, the AEx-ANT was close to AEx-MIO samples, while AEx-PHE was positioned further to the right, as AEx-GTS. However, the 110th cycle narrowed the distance between PAH experiments, while placed in opposite sides for PC1 axe for AEx with FOG residues. The FOG residues had reduced impact in the community evolution after 52 cycles, when compared to anthracene and phenanthrene. The data for fungi community showed a clear separation between cycle 52 and 110, the first mainly due to PC2 dispersion, while the last cycle was separated by PC1 values (Figure 3.10a). The PAH pollutants had lower impact in the fungal community, when compared to the tristearin and lubricant oil effect. The data suggest that the PAH impact overall evolution favouring bacteria, while AEx with FOG pollutants targeted changes in fungi, as shown by the coloured areas in Figure 3.9b and Figure 3.10b. The bacterial OTU with most impact in the positioning of the samples were the *Burkholderia* genus, most determinant for the 110th cycle of AEx-ANT and PHE, the *Acinetobacter* genus, mostly related to AEx-GTS samples and the 52nd cycle of AEx-PHE, and Pseudomonadaceae family and, more specifically, the *Pseudomonas* genus, both associated to the AEx-MIO samples and 52nd cycle of AEx-ANT. On the fungal analysis, the OTUs with most importance for PC were the unclassified Ascomycota and Ascomycota, positively associated with the last cycle of AEx-ANT and GTS, the *Yarrowia* genus positively related to the 52nd cycle of AEx-GTS and MIO, and the unclassified OTU, positively related to the initial WWTP consortium and the 52nd cycles of AEx-ANT and PHE.

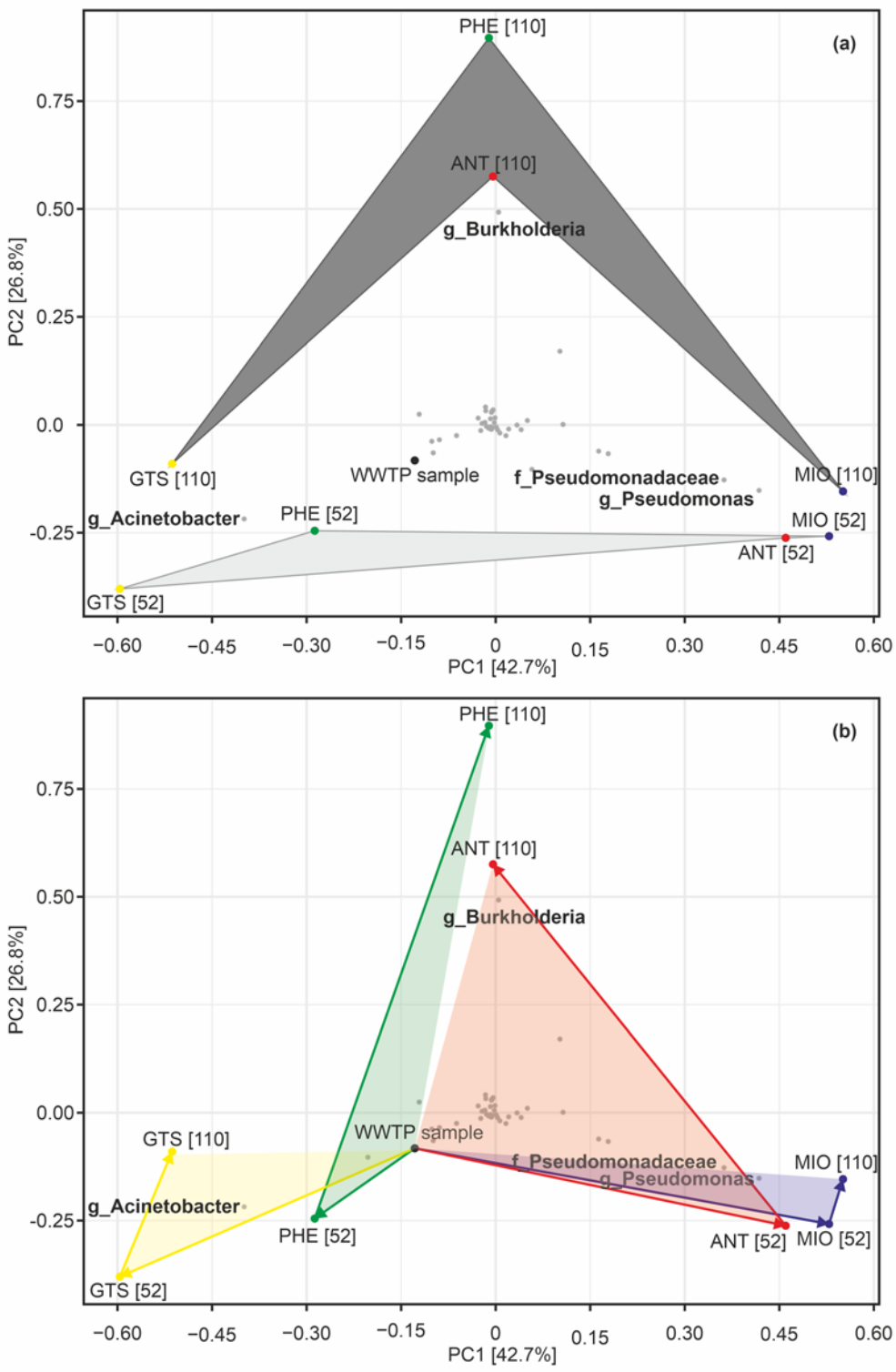


Figure 3.9 – Principal Component Analysis biplot from 16S rRNA gene sequencing taxonomic data. Plot constructed in *AmpVis2* package, representing the two cycles (52nd and 110th) from each AEx and the initial WWTP sample used for starting adaptive evolution experiment inoculation (WWTP sample). Each coloured dot represents a sample, were red stands for anthracene, green for phenanthrene, yellow for stearin and dark blue for mineral oil, while the black dot represents the initial inocula sample. The small grey dots represent the taxonomic assignment contribution for PC results, were the four most impacting taxa are found in bold. The variance retained by each new PC is referred in the label. Two different data relation are depicted: in **(a)** the dots are connected according to cycle number; in **(b)** the dots are connected according to AEx.

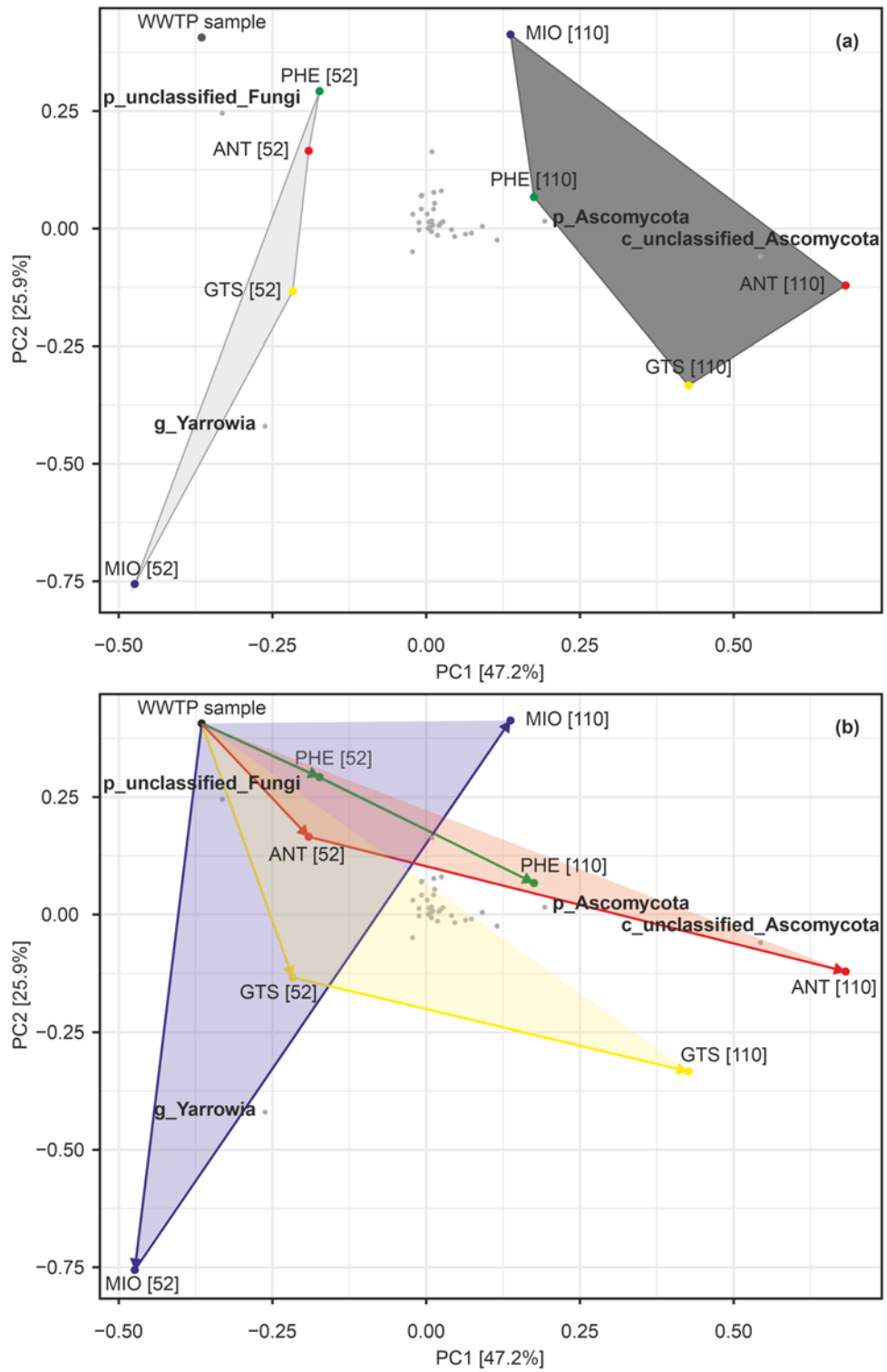


Figure 3.10 – Principal Component Analysis biplot from 26S rRNA gene sequencing taxonomic data. Plot constructed in *AmpVis2* package, representing the two cycles (52nd and 110th) from each AEx and the initial WWTP sample used for starting adaptive evolution experiment inoculation (WWTP sample). Each coloured dot represents a sample, were red stands for anthracene, green for phenanthrene, yellow for stearin and dark blue for mineral oil, while the black dot represents the initial inocula sample. The small grey dots represent the taxonomic assignment contribution for PC results, were the four most impacting taxa are found in bold. The variance retained by each new PC is referred in the label. Two different data relation are depicted: in **(a)** the dots are connected according to cycle number; in **(b)** the dots are connected according to AEx.

While the 52nd cycle samples were predominantly associated to unclassified Fungi and *Yarrowia* genus, especially in AEx-MIO community, the last cycle was most associated to Ascomycota phylum. Unfortunately, the resolution level did not allow to establish beneficial groups and the most impactful class.

3.3.5 Selection of best degraders among representative strains

The clustering of isolates into representative strains allowed the accumulation of different traits, unveiled by the genomic similarity between strains from different AEx. It was possible to select the best degraders among representative strains with higher metabolic plasticity and higher resilience by integrating all the data obtained. On one hand, a representative strain capable of growing with a specific tested substrate, while being able to remain in the highly competitive and stress-induced experiment (i.e. was also isolated at the last adaptive evolution cycle for that substrate), was taken into account. On the other hand, best degraders would display a broader range of interesting traits in terms of biodegradation (i.e. were able to cope with different substrates). Traditional multiple trait dataset representation relies on *Venn* diagrams, which are graphically limited to more than 6 sets. Alternative representation methods, such as *SetUp* plot (Figure 3.11), were developed to cope this limitation, using freely available tools as *R* programming software, with *UpSetR* packages (Lex *et al*., 2014).

In Figure 3.11 are presented all the representative strains and the multiple traits information to allow an integrative analysis of the dataset. Most representative strains (91%) presented 3 or less multiple traits, while only 17 agglomerate 4 or more traits.

The selection of best degraders was based on multiple cumulative traits (associated with at least 3 different pollutants, in terms of AEx and/or isolation medium) and obtained from the 110th cycle. By identifying the position of the representative strains from the 110th cycle, it was possible to select the most evolved and resilient 19 strains, pinpointed in Figure 3.11. The results show that only one representative strain from 110th cycle was found in all AEx substrates and isolated in all selective media, namely BBC|573, and another was also isolated in all selective media and all AEx with exception to AEx-GTS (BBC|617). Further analysis showed that both representative strains were found in previous cycles, the 12th and 29th, which strengthen our hypothesis that these representative strains are the most evolved version available, with high potential for future application in bioremediation solutions. The representative yeast YBC|515, although not obtained from the 110th cycle, was also selected as a best degrader given its cumulative traits and the unique taxonomic group it represents, with such promising results.

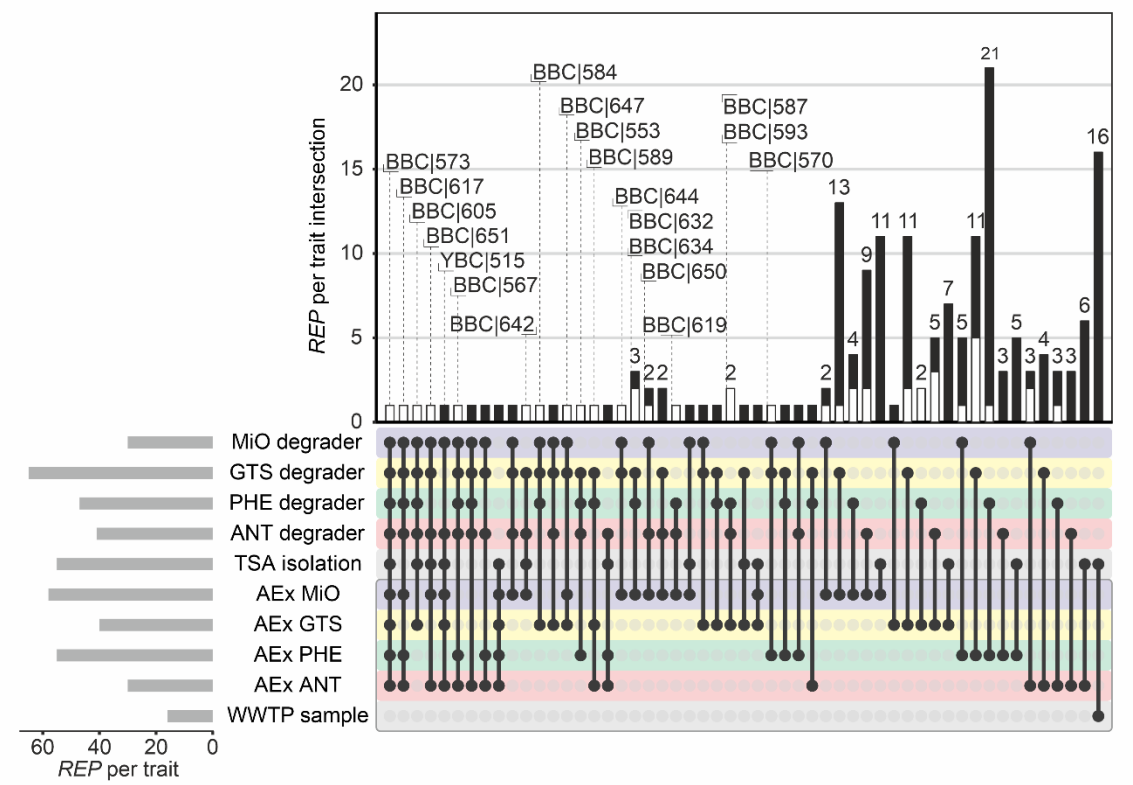


Figure 3.11 – *UpSet* plot for representative strains and cumulative traits for AEx substrate and isolation results. Full dark line and nodes with dot correspond to multiple trait intersection represented in top bar plot, where left to right present less nodes and, consequently, less traits intersection. Top bar plot shows the number of representative strains in each intersection, were white bars symbolize the representative strain from the last isolation cycle (110^{th}). The position of the 19 selected best degraders among representative strains is highlighted by dashed lines. Left bar plot shows the total number of representative strains for each trait set. Traits were divided as: isolation in lubricant mineral oil selective media (MiO degrader); isolation in tristearin selective media (GTS degrader); isolation in phenanthrene selective media (PHE degrader); isolation in anthracene selective media (ANT degrader); isolation in TSA general growth media (TSA isolation); isolation from AEx with lubricant mineral oil (AEx MiO); isolation from AEx with tristearin (AEx GTS); isolation from AEx with phenanthrene (AEx PHE); isolation from AEx with anthracene (AEx ANT); isolation from initial natural inocula (WWTP sample). *REP* stands for number of representative strains.

These cumulative traits indicate a wide metabolic range to degrade the tested substrates: PAH, mineral oil and solid-state triglycerides. Such capacity could be explained by the production of extracellular esterase(s)/lipase(s), while for PAH an extracellular di- or monooxygenase would be required, followed by the necessary intracellular metabolic routes, as previously described.

3.4 Conclusions

In this work, four different adaptive evolution experiments were followed to isolate evolved biodegraders for recalcitrant pollutants (PAH and FOG). By submitting a natural inocula from a biological WWTP, the experimental set-up forced the microbial community to evolve towards the most resilient, degradative and efficient strains, to survive in the harsh environment created. The experiment was maintained for over two years, for 110 cycles of 7

days, and allowed to obtain 455 isolates, that were incorporated into the BBC collection. Selective solid growth media was used to retrieve biodegradative isolates, along the adaptive evolution experiments.

The surveillance over several time-points and in multiple media, supplemented by a fine tune genomic fingerprinting, allowed to disclose a set of 182 genomically distinctive representative strains with high potential for innovative bioremediation products. By analysing the multiple conditions and traits from these representative strains, it was possible to select the most resilient and capable to use most pollutants tested and improved during the AEx.

Furthermore, the profiling of the microbial community showed a dynamic population, evolving separately in all AEx, which proves the potential of adaptive evolution when applied to microbial population. To assess population dynamics, a genomic profiling approach showed the lack of information regarding the Fungi kingdom, suggesting a fast-changing population, common to all AEx, and presumably influencing all other groups. However, the isolation results only retrieved yeasts, which might be explained by the methods used, favouring the bacterial group, or the adaptation of bacteria to grow in solid media, when compared to Fungi. Nonetheless, the results show a clear gap between the culture-dependent and -independent approach, with special impact for the Fungi group.

The selected 19 representative strains stand for the most interesting and valuable assets from the present work, preparing the basis for the continuous effort to create innovative and distinct product for bioaugmentation. The accumulated information, from the phenotypic and genomic information obtained, present valid reasons to further investigate the capabilities of the selected isolates.

Chapter IV – Evaluation and risk assessment of selected PAH and FOG biodegraders

4.1 Introduction

Microorganisms are the primary degraders intervening in the natural decomposition processes. The production of hydrolytic enzymes, targeting environmental pollutants, promotes the bioconversion of recalcitrant compounds to smaller molecules, which can then be incorporate into intracellular metabolic pathways. The polycyclic aromatic hydrocarbons (PAH) are recalcitrant compounds contaminating the environment as a result of human activity. They are produced from the incomplete combustion, mostly engines, and spread through the air, water and soil. The PAHs are included in the organic priority pollutants of great concern for public health due to their toxic effect, genotoxicity, mutagenic and/or carcinogenic properties and their recalcitrance (Khalili *et al*., 1995; European Commission, 2001; Environmental Protection Agency, 2012). These compounds are highly insoluble in water and are considered recalcitrant due to low biodegradation rate measured in the natural environment and extreme low solubility in water.

Many microorganisms, both bacteria and fungi, have been found to degrade PAHs, ranging from low to high molecular weight hydrocarbons (Chakraborty *et al*., 2014). The metabolism of PAHs is largely determined by genetic aspects, given the enzymatic specificity required for xenobiotic degradation. These compounds are usually at low concentrations in the soil and are quite resistant to chemical decomposition, derived from their inherent chemical stability. For most of the low-molecular weight PAHs, both aerobic and anaerobic pathways have been well documented (Cerniglia *et al*., 1992; Chauhan *et al*., 2008; Martínková *et al*., 2009; Seo *et al*., 2009; Mallick *et al*., 2011; Chakraborty *et al*., 2014), with attention focusing greatly on aerobic systems, given its higher efficiency over the anaerobic route (as previously discussed). Briefly, the aerobic degradation of PAHs begins with the action of dioxygenase with the addition of an oxygen atom between two carbon atoms of the benzene ring. The outcome is a *cis*-dihydrodiol and opening of aromatic ring. After several intermediate steps, there is the formation of a dihydroxylated intermediate with hydroxyl groups at positions 1,2 (catechol and protocatechuate) or positions 1,4 (gentisate) by the action of a dehydrogenase enzyme (Figure 4.1a) (Chauhan *et al*., 2008). These common metabolites (catechol, protocatechuate and gentisate) are further degraded through a few central pathways to finally provide intermediates of the citrate cycle, as shown in Figure 4.1b (Martínková *et al*., 2009).

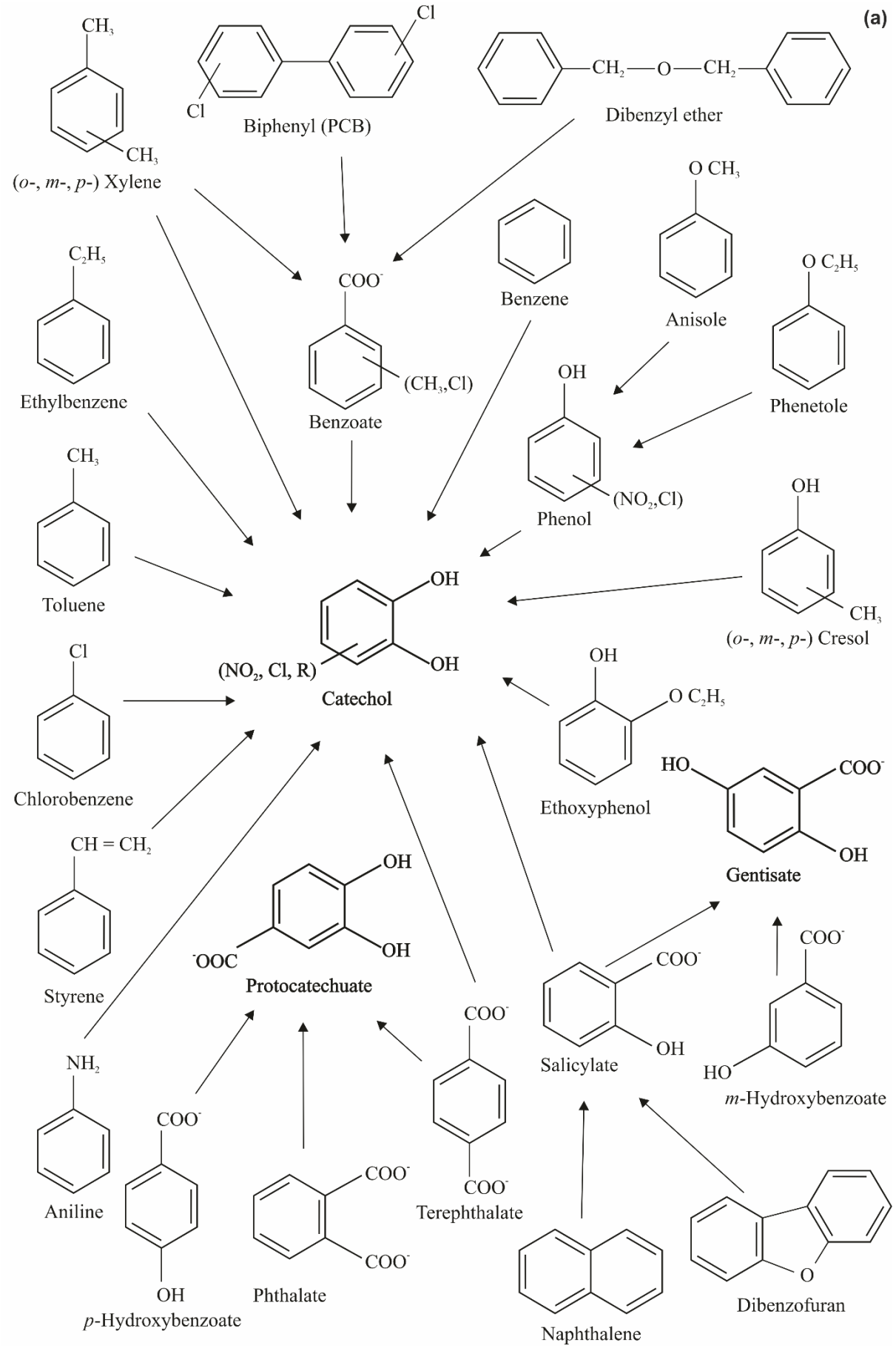


Figure 4.1 (continued)

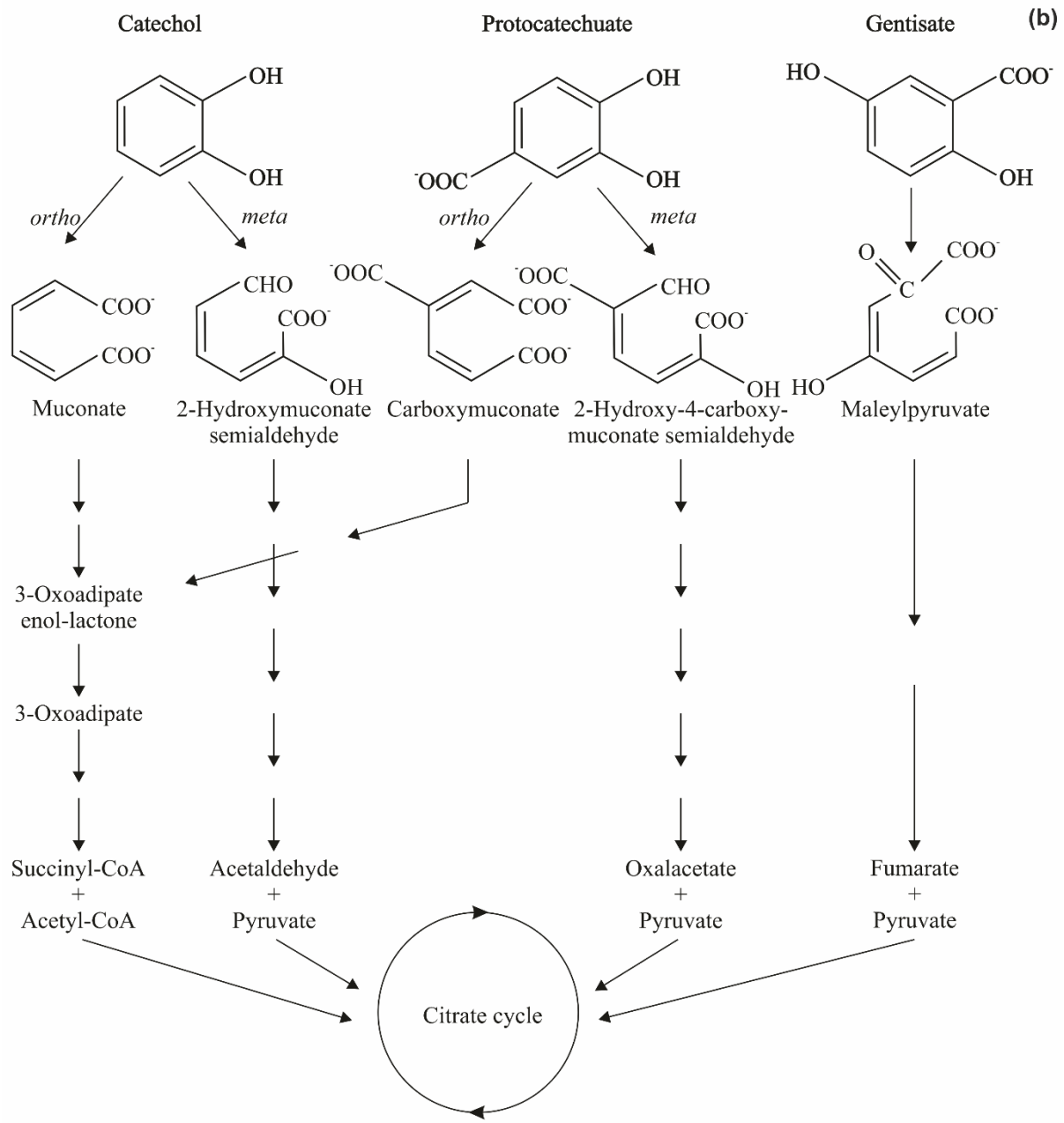


Figure 4.1 – Metabolic pathways for PAH biodegradation in bacteria. **a)** Peripheral pathways of biodegradation of aromatic hydrocarbons to known intermediates (catechol, protocatechol and gentisate). **b)** Central pathways of catabolism of intermediate compounds in bacteria. R=alkyl. Adapt from Martínková *et al*. (2009).

The ring cleavage of both catechol and protocatechuate catalysed by dioxygenases occurs either between the hydroxyl groups (intradiol cleavage or *ortho*-cleavage) or adjacent to one of the hydroxyl groups (extradiol-cleavage or *meta*-cleavage). The *ortho*-cleavage pathway of catechol and protocatechuate converging at 3-oxoadipate is also called the 3-oxoadipate (β-ketoadipate) pathway (Harwood and Parales, 1996). In gentisate, cleavage occurs between the carboxyl group and adjacent hydroxyl group (Dagley, 1971). The central pathways of aromatic catabolism are common in bacteria (Harwood and Parales, 1996). The catechol,

protocatechuate and gentisate dioxygenases play central roles in degradation of aromatic compounds as they catalyse the critical and chemically difficult aromatic ring-cleavage reaction. Quite distinct groups have been found to degrade PAH compounds, as shown in Table 4.1. These include bacteria from diverse phyla as Actinobacteria, with genera *Arthrobacter*, *Brevibacterium*, *Corynebacterium*, *Gordonia*, *Janibacter*, *Micrococcus*, *Mycobacterium*, *Nocardia*, *Nocardioides*, *Rhodococcus*, *Streptomyces* and *Terrabacter*, also Bacteroidetes, with *Chryseobacterium* and *Flavobacterium* (both Flavobacteriia), and Firmicutes, with genera *Bacillus* and *Staphylococcus*. However, major examples come from the Proteobacteria phylum, namely the Alphaproteobacteria, with genera *Beijerinckia* and *Sphingomonas*, the Betaproteobacteria, with genera *Achromobacter*, *Acidovorax*, *Alcaligenes*, *Burkholderia*, *Polaromonas* and *Ralstonia*, and the Gammaproteobacteria, with genera *Aeromonas*, *Cycloclasticus*, *Marinobacter*, *Moraxella*, *Pasteurella*, *Pseudomonas*, *Rhodanobacter*, *Stenotrophomonas*, *Vibrio* and *Xanthomonas*. These groups include both rod- and coccus-shaped cells, Gram-positive and negative, endospore-forming bacteria, making it quite difficult to establish a pattern. This points towards multiple biodegradation pathways for PAH, non-restricted to a specific group, with multiple genera associated to similar compounds. Although some genera have already been associated to multiple targets, as *Pseudomonas* associated to most low-molecular weight PAH degradation, others show limited capacity, as *Aeromonas*, which has only been reported as phenanthrene degrader (Table 4.1). This might result from the low number of studies with unconventional genera (as *Beijerinckia* or *Terrabacter*) in favour of commonly known degrader ones, as *Pseudomonas* and *Mycobacterium*.

The PAH biodegradation pathways are fundamentally determined by the activity of specific enzymes: oxygenases. They can be categorized in both EC 1.13 and EC 1.14, as oxidoreductases, the first for monooxygenases and second for dioxygenases. They carry the incorporation or reduction of molecular oxygen with other substrates, with cytochrome c oxidase as the most studied example. The cytochrome c oxidase is the usual terminal oxidase enzyme in electron transfer chains, for oxidase positive bacteria. In Figure 4.2 are presented the key-point actions for oxygenase activity, particularly important for all hydrocarbon degradation pathways, with enzyme determining the possible pathway for complete PAH incorporation as gentisate, catechol or protocatechol. For example, if phthalate is degraded via salicylate 1-hydroxylase (S1H) instead of salicylate 5-hydroxylase (S5H), then only catechol will be produced. This shows possible improvements towards specific enzymes production or configuration, when tackling similar problems.

Table 4.1 – List of known bacterial species capable of degrading aromatic compounds (from Hamann *et al*., 1999; Samanta *et al*., 2002; Seo *et al*., 2009). ADBT, alkylated dibenzothiophene; ANT, anthracene; BaA, benz[*a*]anthracene; BaP, Benzo[*a*]pyrene; BP, biphenyl; BT, benzothiophene; BZ, benzoate; CBP, chlorobiphenyl; CBZ, carbazole; CDBF, chlorinated dibenzothiophene; CDD, chlorinated dibenzo-*p*-dioxin; CHR, chrysene; DBA, dibenz[*a*,*h*]anthracene; DBF, dibenzofuran; DD, dibenzo-*p*-dioxin; dMBaA, dimethylbenz[*a*]anthracene; FLA, fluoranthene; FLE, fluorene; HFBT, 3-hydroxy-2-formylbenzothiophene; MNAP, methyl naphthalene; NAP, naphthalene; NAT, naphthothiophene; PHE, phenanthrene; PYR, pyrene; TOL, toluene.

Species / strain	Aromatics
***Achromobacter* sp. NCW**	CBZ
***Acidovorax delafieldii* P4-1**	PHE
***Aeromonas* sp.**	PHE
Alcaligenes denitrificans	FLA, NAP, PHE
Alcaligenes faecalis	PHE
Arthrobacter polychromogenes	PHE
***Arthrobacter* spp. F101 and P1-1**	CBZ, DBT, FLE, PHE
***Arthrobacter sulphureus* RKJ4**	PHE
Bacillus cereus	NAP, PYR
***Bacillus* sp.**	PHE
***Beijerinckia* sp.**	PHE
***Brevibacterium* sp. HL4**	PHE
Burkholderia cepacia	BaA, CHR, FLA, NAP, PHE, PYR
Burkholderia cocovenenans	PHE
***Burkholderia* spp. S3702, RP007, 2A-12TNFYE-5, BS3770, and C3**	PHE
***Burkholderia xenovorans* LB400**	BZ, BP
***Chryseobacterium* sp. NCY**	CBZ
Corynebacterium venale	NAP
***Cycloclasticus* sp.**	BaA, CHR, FLA, PYR, NAP
***Flavobacterium* sp.**	BaA, CHR, FLA, PHE, PYR
***Gordonia* sp. Bp9**	PYR
***Janibacter* sp.**	ANT, DBF, DBT, DD, FLE, PHE
***Marinobacter* sp. NCE312**	NAP
***Micrococcus* sp.**	PHE
***Moraxella* sp.**	NAP
Mycobacterium flavescens	FLA, PYR
***Mycobacterium gilvum* DSMZ 9487**	PYR
***Mycobacterium* spp. JS14, 6PY1, KR2, RJGII-135, PYR-1, LB501T, and DSMZ 2966**	ANT, BaA, BaP, CHR, FLA, FLE, PHE, PYR, NAP
***Mycobacterium vanbaalenii* PYR-1**	dMBaA, PHE, PYR
***Nocardia* sp.**	PHE
***Nocardioides aromaticivorans* IC177**	CBZ
***Pasteurella* sp. IFA**	FLA
***Polaromonas naphthalenivorans* CJ2**	NAP
Pseudomonas aeruginosa	BaA, CHR, FLA, PYR, PHE
Pseudomonas cepacia	NAP
***Pseudomonas fluorescens* BS3760 and DSMZ 6505**	BaA, CHR, PHE, BaA, FLA, NAP, PYR
Pseudomonas paucimobilis	NAP, PHE
***Pseudomonas putida* P16, BS3701, BS3750, BS590-P, BS202-P1, CSV86, DSMZ 4302, DSMZ 4476, DSMZ 50222, DSMZ 50208, DSMZ 3931, and DSMZ 3934**	BaA, CHR, FLA, MNAP, NAP, PHE, PYR, TOL
Pseudomonas saccharophilia	PYR
***Pseudomonas* spp. C18, PP2, DLC-P11, BT1d, B4, HH69, CA10, NCIB 9816-4, F274, NCIMB 12229, Dw 2-21, MK Naph I, MK Naph II, and MK Naph III**	ANT, BP, CBZ, CDD, CBP, DBF, DBT, FLE, HFBT, PHE, NAP
***Pseudomonas stutzeri* P15**	PYR
Pseudomonas testosteroni	NAP
Pseudomonas vesicularis	FLE, NAP
***Ralstonia* spp. SBUG 290 and U2**	DBF, NAP
***Rhodanobacter* sp. BPC-1**	BaP
***Rhodococcus erythropolis* I-19 and D-1**	ADBT, DBT
***Rhodococcus* sp.**	BT, CHR, FLA, PHE, PYR, NAP, NAT
***Sphingomonas paucimobilis* EPA505, Ba II, DSMZ 1098, DSMZ 6900, PF I, P2, and LB126**	ANT, BP, CBZ, DBF, DBT, FLA, FLE, PHE, NAP
***Sphingomonas wittichii* RW1**	CDD
***Sphingomonas yanoikuyae* R1 and JAR02**	BaA, BaP, CHR, FLA, PYR
***Staphylococcus* sp. PN/Y**	PHE
***Stenotrophomonas maltophilia* VUN 10 010, VUN 10 003, MK Phe, MK Anth I, MK Anth II**	ANT, BaA, BaP, COR, DBA, FLA, NAP, PHE, PYR
***Streptomyces* sp.**	NAP and PHE
***Terrabacter* sp. DBF63**	CDBF, CDD, DBF, FLE
***Vibrio* sp.**	NAP and PHE
***Xanthomonas* sp.**	BaP, CBZ, PYR

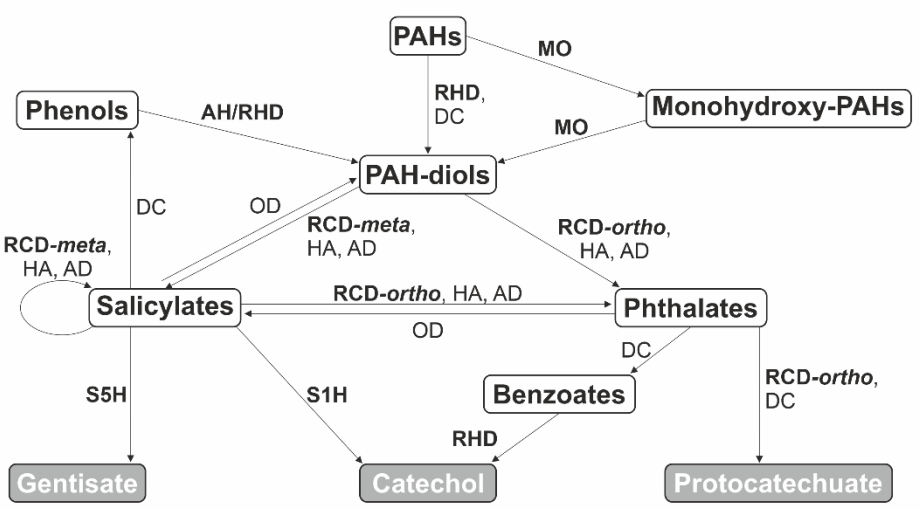


Figure 4.2 – Schematic view on the roles of various oxygenases in guiding diverse metabolic pathways in the bacterial assimilation of PAHs. Oxygenases involved are shown in bold font: RHD, various classes and subclasses of ring-hydroxylating dioxygenases; OD, oxidative decarboxylase; MO, monooxygenase (possibly cytochrome P450-dependent monooxygenase), AH, aromatic hydroxylase; S1H, salicylate 1-hydroxylase; S5H, salicylate 5-hydroxylase (belong to the family of RHD); RCD, various classes and subclasses of *-meta* or *-ortho* ring cleavage dioxygenases, where the substrates are either catecholic or salicylate-type compounds. Other enzymes involved are shown in normal font: HA, hydratase-aldolase; AD, aldehyde dehydrogenase, DC, decarboxylase. Enzymes like isomerases that are reported to be involved in low molecular weight PAHs degradation pathways have not been included. Adapt from Mallick *et al*. (2011)

The knowledge given by metabolic pathways and its interactions has shown the potential implications for improvement and design of novel full-scale biological systems. Regarding the bioremediation of lipids, a commonly used abbreviation in this field of activity is FOG. This expression regards the indiscriminate quantification method used for FOG determination, which considers every compound soluble in n-hexane as a lipid content. Moreover, such nomenclature expresses the inclusion of both lipid compounds (the fatty and oil residues) and also the less soluble compounds from butter and animal fat, as grease, and also the synthetic oils used, usually derived from alkanes. All these mixtures can be assessed by the FOG determination method.

Considering microbial metabolism, it is fundamental to establish substrates according to chemical proximity and similarity of degradation pathway. Looking at the lipidic content, it is usually divided in triacylglycerol (TAG), diacylglycerol (DAG) and free fatty acids (FFA), from various lengths, depending on the original sources, as previously discussed. However, only FFAs can be directly transported into cells, which means that the TAG and DAG must first be broken down by the action of an extracellular lipase or esterase for FFA release, as shown in Figure 1.5. The fatty acids are divided according to its length, determined by the number of carbons, as long-chain fatty acids (LCFAs, C_{12}-C_{18}), medium-chain fatty acids (C_7-C_{11}) and short-chain fatty acids (SCFA, C_4-C_6). While LCFA require facilitated transport to

enter cells, short and medium-chain FFAs can enter the cell by free diffusion or by other unknown mechanism (Nunn *et al*., 1979; Maloy *et al*., 1981; Black and DiRusso, 1994). It has also been proposed that porin OmpF may facilitate the transport of SCFA (Rodríguez-Moyá and Gonzalez, 2015). The incorporation of LCFA has been described for *E. coli* as highly dependent on the fatty acid transport protein (FadL), located in the outer membrane and acting as a LCFAs receptor, as well as on acyl-CoA synthetase (FadD, EC 6.2.1.3), in the inner membrane and the cytosol intracellular components (Figure 4.3). A variety of FadL homologue proteins have been found in Gram negative bacteria, and also involved in xenobiotic and alkane uptake (Jimenez-Diaz *et al*., 2017).

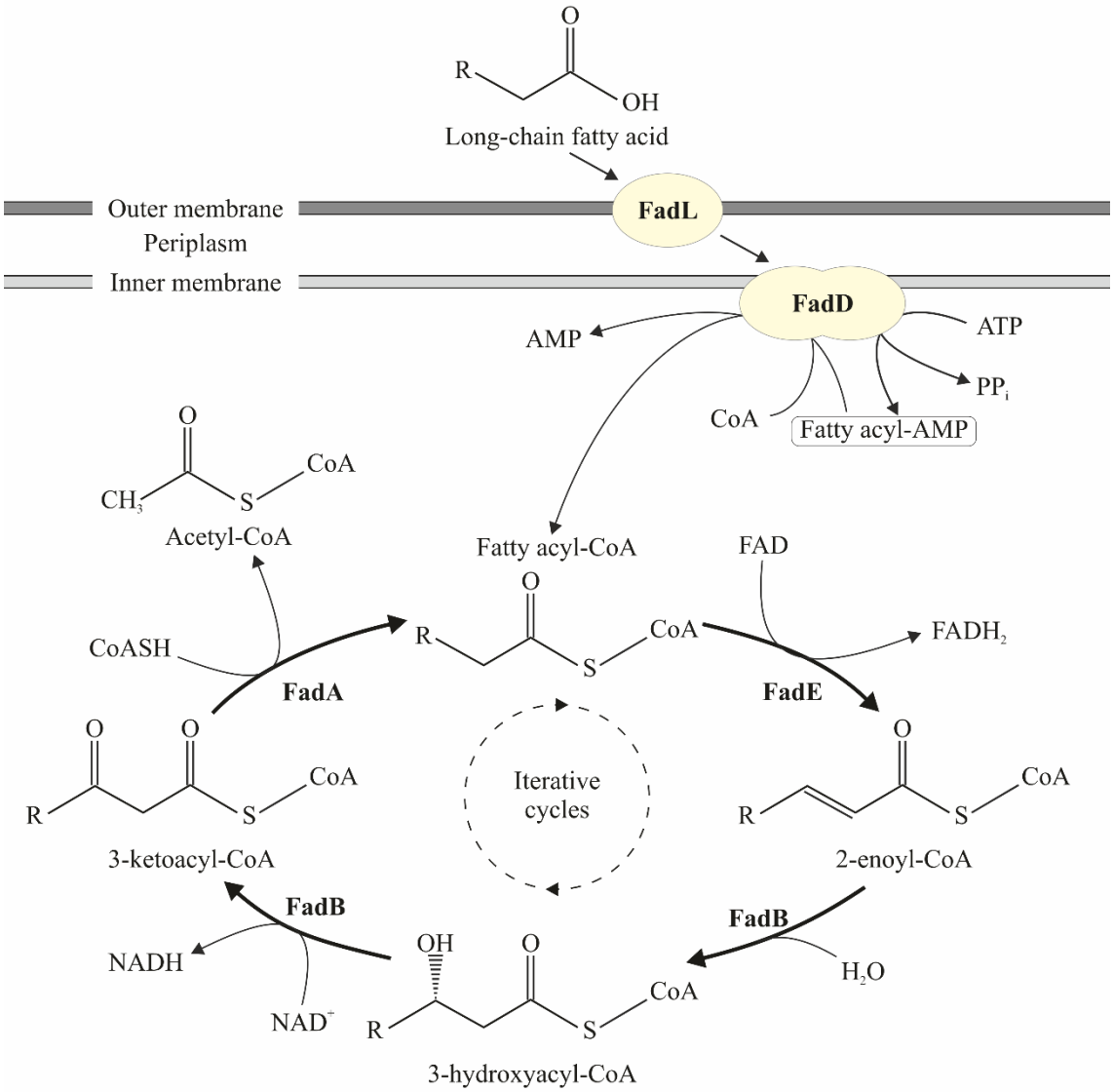


Figure 4.3 – Schematic representation of the fatty acid degradation pathway in gram-negative bacteria. The FadL represents a transporter protein for LCFA, facilitating the passage to the periplasm of FFA. The FadD enzyme complex catalyses two reactions, consuming an ATP and a coenzyme molecule to produce a fatty acyl-CoA to enter the cell. The fatty acyl-CoA can enter the *β*-oxidation reactions, which shortens the fatty acid molecule by two carbons in each iterative cycle. Adapted from Jimenez-Diaz *et al*. (2017)

As illustrated, FadD converts FFA transported from FadL into fatty acyl-CoA in a two-step reaction: first a pyrophosphorolysis of ATP, linking the carboxyl group of FFA by an acyl bond to phosphoryl group of AMP, liberating the P_i from ATP; then the fatty acyl is bonded to the coenzyme A with the liberation of the AMP group (Figure 4.3). From here, the fatty acyl-CoA can start the β-oxidation cycle, by the fatty acid *β*-oxidation multienzyme complex (FadAB), with the production of one molecule of $FADH_2$, NADH and acetyl-CoA, shortening the fatty acyl-CoA by two carbons for each cycle. Although less common, unsaturated fatty acids can also be degraded using the same pathway. For the degradation of FFAs with double bonds located in odd-numbered carbons, a 3,2-enoyl-CoA isomerase activity must first act to allow the unsaturated fatty acyl-CoA to enter the β-oxidation cycle (Pramanik *et al.*, 1979; Clark and Cronan, 2005). In the case of unsaturated FFA in even-numbered carbons, a further enzyme (2,4-dienoyl-CoA reductase) is required to remove the double bond, in order to allow the process to continue (Jimenez-Diaz *et al.*, 2017).

The described metabolic pathway shows the possible route that microorganisms, specially bacteria, may follow to consume the target pollutants produced and accumulated in the environment from human activity. The biological approach to deal with PAH and FOG accumulation must present rational and scientific background confirmation to facilitate comprehension about the biochemical interactions followed by microorganisms to degrade these compounds. However, both the above classes present an increasing problem: very low solubility in water. This feature makes theses substrates harder to analyse and test at laboratory scale, and even more at full-scale approaches. Also, the activity of microorganisms is enhanced in environments with higher water content, by facilitating nutrients uptake, increasing the transportation of metabolites, and the diffusion of toxic by-products. Nevertheless, these are recalcitrant compounds, accumulating in the environment with possible harsh effects on the human health, and a biological approach can help to detoxify or at least reduce the presence of such pollutants.

In the Chapter 4 are presented the results of the degradation capacity of the previously selected microbial strains, for lipids and hydrocarbons biodegradation. An initial genetic screening for metabolic relevant genes was performed, to assess possible enzymatic pathways. Furthermore, the selected isolates reached higher degradation ability for lipid residues degradation, when compared to commercially available products, at laboratory level. The promising results show great improvement of available biological products, when applying real wastewater FOG-separator residues. Also, the selected strains for hydrocarbons degradation were able to effectively remove PAH residues, in just a few days. Such results

support our confidence in the previously selected strains, to develop new and improved bioaugmentation products for bioremediation purposes targeting recalcitrant pollutants such as PAH and triglycerides.

4.2 Materials and Methods

4.2.1 Microbial strains

All the microorganisms studied in this chapter were previously isolated, whether directly from WWTP effluent samples or from laboratory adaptive evolution experiments (AEx). The strains were screen for their biodegradation ability for PAH and/or FOG residues, leading to their selection for further characterization, as described in this chapter. In Table 4.2 are summarized the information of the strains studied (20 bacteria and 1 yeast), with regard to the isolation source and selective media. The analysis of these strains was extended by the search of genetic elements involved in known PAH metabolic pathways, monitoring the cell growth in mineral medium with pollutants as sole carbon source, and the degradation ability for PAH and/or FOG compounds. For the assays involving PAH metabolism, all selected strains from AEx were tested, given their natural source and/or isolation media. In the case of the yeast strain YBC|515, it was used as a negative control for the search of genes evolved in PAH metabolism, given the bacterial origin of the targeted genes for PCR amplification. For the FOG degradation assays, strains BBC|043 and BBC|148, selected for their ability to degrade oleic acid and triolein (Chapter II), were also included. Three strains from AEx origin (BBC|570, BBC|584 and BBC|644) could not be evaluated for FOG degradation. All the strains studied presented previous results with potential application for bioremediation approaches, which were further evaluated.

4.2.2 Detection of genes involved in PAH degradation pathways

The search for genes involved in the degradation pathway for PAH was performed by PCR amplification using previously described primers targeting multiple bacterial groups (Sei *et al*., 1999; Cébron *et al*., 2008; Izmalkova *et al*., 2013). In Table S1 are listed the primers sequence in detail, for each reaction: for ring-hydroxylating dioxygenase coding gene, the pair PAH-RHD (GPf/GPr and GNf/GNr, for Gram positive and Gram negative bacteria, respectively); for catechol 1,2-dioxygenase coding gene, the pair C12Of/C12Or; for catechol 2,3-dioxygenase coding gene, the C23Of/C23Or primers; and for salicylate 5-hydroxylase coding gene, the sgp319f/1238r primers.

Table 4.2 – List of previously selected strains studied in Chapter IV. The biological source refers to original isolation inoculum, divided in native (for strains from WWTP natural samples) and adaptive evolution experiments (AEx), where full coloured circle specify the previous AEx: red for AEx-ANT, green for AEx-PHE; yellow for AEx-GTS and dark blue for AEx-MIO. The selective media specifies the pollutant used as sole carbon source in the selective isolation, represented by full coloured squares: red for anthracene, green for phenanthrene; yellow for tristearin and dark blue for mineral lubricant oil. The assays correspond to the experimental approaches in Chapter IV, signalling where each selected strain was applied (green mark) or not (red cross).

Strain	Biological Source	Selective Media	Assays			
			Genes for PAH degradation	Microplate growth	Hydrocarbon degradation	FOG degradation
BBC|043	Native	*na** *na**	✗	✗	✗	✓
BBC|148	Native	*na** *na**	✗	✗	✗	✓
BBC|553	● ●	■ ■ ■	✓	✓	✓	✓
BBC|567	● ●	■ ■ ■ ■	✓	✓	✓	✓
BBC|570	●	■ ■	✓	✓	✓	✗
BBC|573	● ● ●	■ ■ ■ ■	✓	✓	✓	✓
BBC|584	●	■ ■ ■	✓	✓	✓	✗
BBC|587	●	■ ■	✓	✓	✓	✓
BBC|589	● ●	■ ■	✓	✓	✓	✓
BBC|593	●	■ ■	✓	✓	✓	✓
BBC|605	●	■ ■ ■	✓	✓	✓	✓
BBC|617	● ● ●	■ ■ ■ ■	✓	✓	✓	✓
BBC|619	●	■ ■	✓	✓	✓	✓
BBC|632	●	■ ■	✓	✓	✓	✓
BBC|634	●	■ ■	✓	✓	✓	✓
BBC|642	●	■ ■ ■	✓	✓	✓	✓
BBC|644	●	■ ■	✓	✓	✓	✗
BBC|647	● ●	■ ■ ■	✓	✓	✓	✓
BBC|650	●	■ ■	✓	✓	✓	✓
BBC|651	● ●	■ ■ ■	✓	✓	✓	✓
YBC|515	● ●	■ ■	✗	✓	✓	✓

* *not assessed*

The amplification reactions were performed in a T1 Thermocycler (Biometra), using a total volume of 50 µL and conditions varying for each pair of primers, following the initial publications of their description. For the ring-hydroxylating dioxygenase coding gene, the PCR reaction used 1X reaction buffer, 1.2 U of TaqDNA polymerase, 0.2 mM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 0.2 µM of primer and 1 µl of DNA extract. The reactions for the remaining genes were slightly different: 1X reaction buffer, 1 U of TaqDNA polymerase, 0.2 mM of each deoxynucleoside triphosphate, 2 mM $MgCl_2$, 0.5 µM of primer and 1 µl of DNA extract. The PCR cycling conditions for the ring-hydroxylating dioxygenase coding gene were: 95°C for 5 min followed by 30 cycles of 30 s at 95°C, 30 s at 54°C (for GPf/GPr) or 57°C (for GNf/GNr) and 30 s at 72°C, plus one additional cycle at 72°C for 7 min. For the PCR amplification reactions of catechol dioxygenase coding genes, the conditions were: 95°C for 5 min, followed by 40 cycles of 30 s at 94°C, 30 s at the annealing temperature and 30 s at 72°C.

Considering that each C12O and C23O target sequence had a different homology score with the primers, a touch-down method was employed. For C12O, the annealing temperature was 61°C in the first 10 cycles followed by a step down to 59°C in the next 15 cycles and 57°C in the last 15 cycles while for C23O, the annealing temperature was 59°C in the first 10 cycles followed by 57°C in the next 15 cycles and 55°C in the last 15 cycles The final step consisted of 72°C for 7 min. For the primers targeting the salicylate 5-hydroxylase coding gene, the PCR conditions were: 95°C for 5 min followed by 30 cycles of 45 s at 94°C, 30 s at 55°C and 90 s at 72°C, plus an additional cycle at 72°C for 7 min. PCR product profiles were visualized (10 µl) after electrophoresis (120 V for 1 h) in agarose gel (1.5% w/v) with 0.5X TBE buffer and staining with ethidium bromide. The 1 kbp DNA Ladder Plus (Invitrogen, UK) was used as a DNA molecular mass marker.

4.2.3 Microplate growth assay using pollutants as sole carbon source

The growth ability of the selected strains in the presence of the tested pollutants was evaluated in a microplate assay. Each well contained 190 µL of mineral medium M9 (see Chapter 2 for composition), supplemented with 0.1% w/v of anthracene, phenanthrene, tristearin or lubricant oil as sole carbon source to test growth ability, or only with medium M9 to assess viability loss over time. The microplates were incubated at 28°C for 15 days, after inoculation with 10 µL of a cell suspension with 1 optical density (OD), in duplicates.

Since measurements commonly used to follow microbial growth, as OD, could not be applied due to interference of the solid substrates used as carbon source, a strategy was set up to

determine the most probable number (MPN) in liquid medium, using Tryptone Soy Broth (TSB, from Biokar Diagnostics, France). The MPN is a statistical method used to estimate the viable numbers of microorganisms in a sample by inoculating broth in 10-fold dilutions and is based on the principle of extinction dilution. It is often used in estimating viable cell number in water and food. For an integrative analysis of all obtained data, the complete dataset of MPN (300 values, from 19 strains and the uninoculated control, in 5 different conditions, for 3 time-points) was examined by Principal Component Analysis (PCA), after logarithm transformation (base-10), using NTSys software (Rohlf, 2008).

4.2.4 PAH extraction and quantification method

Different methods have been proposed for PAH extraction, focusing on the higher affinity of such compounds with organic solvents. For the present work, a liquid-liquid extraction (LLE) was tested, applying acetonitrile (Carlo Erba) as the extraction solvent. The quantification method was tested in glass tubes containing 10 mL solution with known concentrations, extracted with 10 mL of acetonitrile, followed by sample filtration with 0.2 µm pore size filter prior to evaporation step, and resuspension in equal volume of acetonitrile solution (50% v/v), before quantification analysis by high performance liquid chromatography (HPLC). The filtration step intended to remove cell debris which could interference with further analysis. The HPLC equipment (Beckman Coulter) was equipped with a C18 reverse phase column (5 µm particle size, 25 cm length, 4.6 mm internal diameter, Supelco), with affinity for PAH compounds. A 10 µL sample was eluded at a constant flowrate (1 mL/min), using an acetonitrile: water gradient program as follows: isocratic 60:40 for 5 min, linear gradient increase to pure acetonitrile for 20 min (Gratz *et al*., 2011). The concentration of each PAH was calculated from a standard curve based on peak area from a fluorescence detector (Jasco FP-2020) equipped with a data acquisition software (32 Karat) at 280 nm excitation wavelength and 355 nm emission wavelength. Several quantification parameters were determined for the quantification method, namely: accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ). The accuracy was determined by the coefficient of variation (CV) of analysing a sample of known concentration, a reference solution, multiple times (Andersen and Skovgaard, 2010). The precision was expressed as the percentage of recovery of the expected value, also from a reference solution. The LOD and LOQ were defined as the lowest concentration at which the method was able to detect or quantify the samples, with the noise to signal ratio as 1:3 for LOD and 1:10 for LOQ, following the equations 4.1 and 4.2:

$$LOD = \frac{|a| \pm 3S_a}{b}$$

Equation 4.1

$$LOQ = \frac{|a| \pm 10S_a}{b}$$

Equation 4.2

Where the $|a|$ stands for the absolute value of the intercept in the linear predictor, S_a for standard deviation of a, and b for the regression coefficient of the linear regression model used (Rao, 2018).

4.2.5 Hydrocarbon biodegradation assays

Biodegradation experiments for PAHs were carried out using glass tubes with screw stopper (Duran) containing 5 mL of M9 mineral medium and PAH (1 g/L) as sole carbon source. The PAHs used in these assays were anthracene and phenanthrene (Sigma Aldrich), separately. A total of 24 strains (in Table 4.2), previously selected by indirect observation of PAH consumption, were tested to quantify PAH reduction by whole-cell analysis. The inoculum was prepared from previously grown biomass, in TSA medium over-night at 28°C, by suspending a colony in physiologic saline solution (0.8% w/v sodium chloride). The optical density of cells was adjusted to a final 0.1 OD, after inoculation, to allow direct comparisons of total PAH removal. The tubes were incubated for 7 days at 28°C without shaking, with an abiotic control, without inoculum, and all assays were performed in triplicates. After 7 days, the whole PAH content was extracted by LLE with 5 mL acetonitrile, and quantified by HPLC method, as previously described. Given that there was no filtration before the LLE, the degradation values obtained refer to actual PAH catabolism, not caused by simple cellular incorporation or adherence to cell wall.

4.2.6 FOG extraction and quantification method

A FOG extraction and quantification method was developed during the present work, based on the EPA method 1664 (USEPA, 2010). This method uses *n*-hexane as a solvent for LLE, followed by a polar phase separation, collection and evaporation, allowing the quantification of the solid residue retrieved after drying of the organic phase. For the present work, conical 50 mL tubes were used, with 40 mL M9 media supplemented with known concentration of oily substrate (oleic acid, triolein or other). After addition of 10 mL *n*-hexane and vigorous shaking and vortex for 2 min, the samples were centrifuged at 3,220 X g for 15 min, and the upper polar phase was collected to a previously weighed glass tube. This extraction was repeated at least twice and collected for the same glass tube. The samples were evaporated under pressure (160 mBar) and temperature (50°C) for at least 3 h. This protocol was

developed after incongruent results for pure substrate extraction (oleic acid and triolein) without centrifugation in triplicate biological samples (data not shown). The quantification parameters were determined following the same principles applied for FOG quantification method 1664.

4.2.7 FOG biodegradation assays

The FOG biodegradation assays were carried out in Erlenmeyer flasks, with 40 mL M9 media supplemented with oily residues, from a real FOG separator, as sole carbon source. The assays were carried at 28°C, with 150 rpm continuous shaking, for 5 days. Selected strains from different isolation experiments were tested, as well as two commercial products for comparison. The cell viability was followed by the MPN method, as previously described. Prior to the whole sample LLE, a 100 µL sample was collected for viability determination. All assays were conducted in triplicates, and for multiple time-point analysis, a second set of flasks were inoculated from the same inoculum and maintained at the same conditions. This was necessary given the insoluble nature of the carbon source and the inability to quantify the FOG content without a whole-sample extraction. Given that there was no filtration before the LLE, the removal values obtained refer to actual FOG consumption and not caused by simple cellular incorporation or adherence to cell wall or simple transformation into similar compounds.

4.2.8 Molecular identification of selected strains

Identification of selected strains at species level was achieved by sequencing of 16S rRNA gene. Primers pA and 1392R were used for amplification (Lane, 1991). PCR reactions were carried out as described for single primer PCR-fingerprinting in Chapter 1. PCR fragments were sequenced in GATC Biotech AG laboratory (Cologne, Germany), and species allocation was achieved by homology search in GenBank database at NCBI (National Center for Biotechnology Institute), using BLAST algorithm (Altschul *et al*., 1990).

4.3 Results and Discussion

4.3.1 Putative metabolic pathway through genetic elements

The search for key enzymes involved in PAH catabolic pathways was performed through PCR detection using specific primers targeting genes coding for ring-hydroxylating dioxygenase for Gram positive and Gram negative bacteria, catechol 1,2-dioxygenase (2,

primers C12Of/ C12Or), catechol 2,3-dioxygenase (3, primers C23Of/ C23Or) and salicylate 5-hydroxylase (4, primers sgp319f/123r) (See Table S1 for details).

The approach intended to unveil the main metabolic pathway the strains could follow: whether by dioxygenase or monooxygenase initial attack, by the presence of a ring-hydroxylating dioxygenase gene; the catechol dioxygenase genes would answer the cleavage position for catechol incorporation (-*ortho* for catechol 1,2 dioxygenase or -*meta* for catechol 2,3 dioxygenase); and if salicylate was an available substrate, if it could be converted to gentisate by salicylate 5-hydroxylase. The search of genetic elements from PAH metabolic pathway are summarized in Table 4.3, mostly with negative results. Both used dioxygenase primer sets could not retrieve any positive result, for Gram positive and Gram negative bacteria. They could indicate the presence of unique ring-hydroxylating dioxygenase, present in these strains, or with enough differences for mismatch. Also, in Cébron and colleagues (2008) work, these primers were successful in retrieving genes from strains belonging to *Comamonas*, *Pseudomonas* and *Ralstonia* genera (gram negative bacteria) and *Mycobacterium* genus (gram positive bacteria) from environmental samples, while the analysed strains belong to further genera (as *Acinetobacter*, *Ochrobactrum* or *Sphingobacterium*).

Table 4.3 – Results for the search of functional genes in the 18 previously selected strains from adaptive evolution experiments (AEx). The dioxygenase corresponds to both pair of primers for Gram positive and Gram negative bacteria. Only fragments with the expected size were considered positive results, assigned by the green check mark. See Figure S1 in Supplement Information for detailed information.

Gene coding for	BBC|553	BBC|567	BBC|570	BBC|573	BBC|584	BBC|587	BBC|589	BBC|593	BBC|605	BBC|617	BBC|619	BBC|632	BBC|634	BBC|642	BBC|644	BBC|647	BBC|650	BBC|651	Neg. Cont
Dioxygenase	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
Catechol 1,2 oxygenase	✗	✓	✗	✓	✗	✓	✓	✗	✗	✗	✗	✗	✓	✗	✗	✗	✗	✓	✗
Catechol 2,3 oxygenase	✓	✓	✗	✓	✗	✗	✓	✗	✗	✓	✗	✗	✓	✗	✗	✓	✓	✗	✗
Salicylate 5-Hydroxylase	✗	✗	✗	✗	✗	✗	✗	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗

On the other hand, several strains presented both catechol dioxygenase enzymes, as the case for BBC|567, BBC|573, BBC|589 and BBC|634. The obtained amplicons were of the expected size for catechol 1,2 dioxygenase and catechol 2,3 dioxygenase (282 bp and 380 bp, respectively) (Sei *et al*., 1999). Several reports refer species with both catechol dioxygenases, as *Acinetobacter defluvii*, *Pseudomonas stutzeri*, or *Pigmentiphaga* sp. (Brunet-Galmés *et al*., 2012; Chen *et al*., 2018), derived from whole genome sequencing data. On the other hand, in

a few strains only one of the catechol dioxygenases were detected, as BBC|587 and BBC|651 for catechol 1,2 dioxygenase and BBC|553, BBC|617, BBC|647 and BBC|650 for catechol 2,3 dioxygenase. These results suggest a specific catechol catabolism route, towards *ortho*-cleavage for the first enzyme, or the *meta*-cleavage for the second one, as previously discussed. The salicylate 5-hydroxylase gene was only found in BBC|593, a *Pigmentiphaga* sp. strain, with an expected amplicon of 920 bp (Izmalkova *et al*., 2013). Tittabutr and colleagues (2011) reported several salicylate 5-hydroxylase genes in different species, including a *Pigmentiphaga* sp.. Although not delimiting, the presence of such gene indicates this strain is capable to follow the gentisate route, used for salicylate biodegradation.

Baldwin and colleagues (2003) have proposed similar approach to search and identify relevant genetic elements involved in PAH metabolism, for environmental samples. These technologies might be useful to disclosure novel activities or, coupled with real-time PCR, could help to quantify activities and/or expression factors with vital importance. The presence of such specific enzymes, involved in xenobiotic catabolism and PAH metabolization, strengthens our confidence in the capacity and versatility of the selected strains for biodegradation purposes. The negative results could be caused by unspecific or mismatch sequence, given the broad taxonomic spectrum used in this study. Also, the inability to amplify these genes may refer to specific genetic changes, retained during the adaptive evolution experiments from where the strains were isolated.

4.3.2 Integrated analysis of microplate growth assays

The previously selected strains obtained from the AEx were studied for their capacity to grow in a mineral medium with the tested pollutants as sole carbon source, using microplate growth assays and assessment of viable cell number by MPN method. A Principal Component Analysis (PCA) of the base 10-logarithm of MPN data obtained for the 19 strains in five conditions (dataset of 300 values) was used for an integrative analysis. The data showed that all strains distanced from the negative control (Figure 4.4), derived from the viability maintained over each conditions and time point. As seen in the correlation circle (Figure 4.4b) most conditions were only positively correlated to PC1, with exceptions for growth with anthracene in both 7^{th} and 15^{th} day time-point, negatively correlated to PC2. For this reason, the strains positioned in the fourth quadrant (Figure 4.4a) refer to the strains with overall best results, namely, YBC|515, BBC|570, BBC|573, BBC|567, BBC|642, BBC|650, BBC|619, BBC|632 and BBC|584.

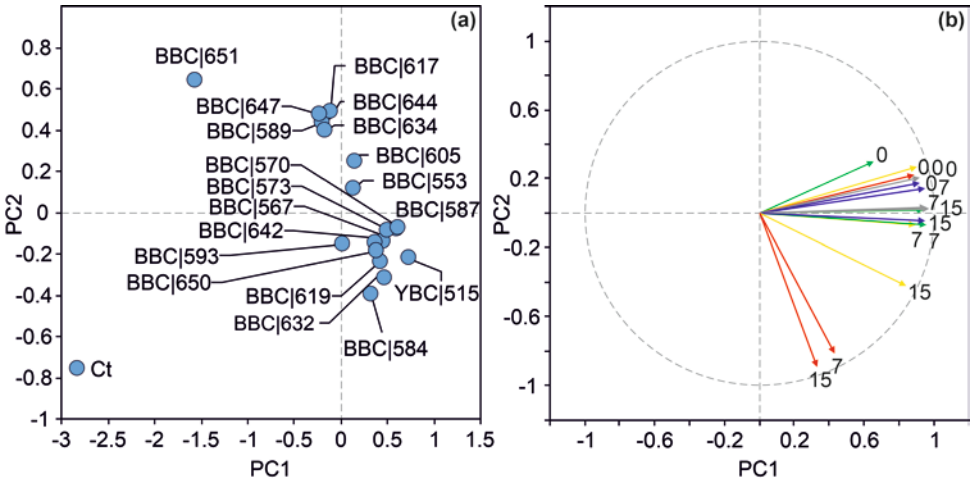


Figure 4.4 – The Principal Component Analysis (PCA) of the base-10 logarithm values of MPN dataset obtained from the growth of 19 strains in minimal medium with different pollutants as sole carbon source, for 15 days. PC1 and PC2 refer to the first two principal components, accounting for 72.9% and 13.1% of total variance, respectively. (**a**) The distribution of strains along the principal components PC1 and PC2. (**b**) The correlation circle including all variables is depicted. Each colour refers to a growth condition: grey without pollutant, red with anthracene, green with phenanthrene, yellow with tristearin and dark blue with lubricant oil. The numbers correspond to the time of incubation, as 0, 7 and 15 days, respectively. *Ct* stands for negative control.

As expected from previously obtained data, the strains were capable to grow or keep their viability in multiple substrates, pointing to possible capacity for future application in bioaugmentation. Several authors have proposed microtiter and microplate methods for fast, high-throughput and inexpensive analysis of biodegradation (Tvrzová *et al*., 2006; Bailes *et al*., 2013; Herzog *et al*., 2014; Lu *et al*., 2015; Meerbergen *et al*., 2018). Typically, these approaches serve to initially screen hundreds of bacterial candidates, to select the most promising ones for further analysis. However, these are indirect methods and effective degradation results are required to prove the efficiency of the selected strains. for removal of recalcitrant compounds. In the present work, given the selection method used to hand-pick the strains from the AEx, as described in Chapter 3, the microplate approach provided proof of cellular growth of these strains, before testing in effective biodegradation assays for PAH and FOG.

4.3.3 Hydrocarbon biodegradation assays

The quantification of PAH can be conducted by different methodologies, some unspecific as gravimetry, or highly precise as chromatography (HPLC, GS, or GS-MS). The HPLC was selected as one of the most fast, accurate and reproducible approaches, allowing the analyses of several hundred samples with a straightforward procedure. Due to some technical differences with procedures adopted by several authors, a validation step was performed to ensure the method was robust and suitable for the evaluation of biodegradation of strains. As

seen in Figure 4.5, anthracene and phenanthrene were quantifiable, with increasing relative variance in higher PAH concentration and a much higher sensitivity. The signal sensitivity was higher for phenanthrene than anthracene, possibly due to the excitation wavelength used. The fluorescence detector was set to 355 nm, which was closer to the optimal fluorescent spectrum value for phenanthrene (364 nm) when compared to anthracene (375.5 nm; Taniguchi and Lindsey, 2018).

There was a clear linear response, with values of the coefficient of determination (R^2) above 99%, as shown in Table 4.4, for quite low detection range (from 10 µg). The calculated values for both LOD and LOQ showed that the method is suitable for the quantification of PAH, if the analysed samples were kept between these values. The calculated accuracy also showed good results, with recovery values very close to 100%, although the precision of the method should be improved, when compared to other authors.

The precision results (14% for ANT and 10% for PHE) could be explained by the extraction step, prior to the HPLC detection and quantification. In fact, previous authors have discussed the variability in the case of PAH quantification and, even in the guidelines, a considerable high variation value is assumed for this analytical method (USEPA, 2010). The selected strains, previously straind from AEx and further analysis, were applied in a biodegradation assay for PAH removal, at laboratory scale (see 4.2.5).

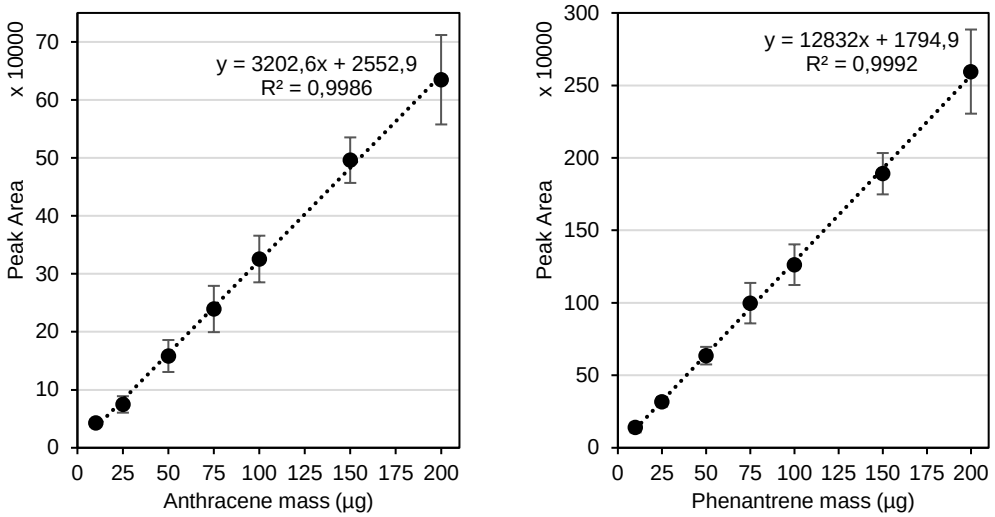


Figure 4.5 – Calibration curves with linear regression for anthracene and phenanthrene. In the linear regression, the points represent a mean of the measures with the respective standard deviation. The linear regression equation and the coefficient of determination are also depicted.

Table 4.4 – Parameters for anthracene and phenanthrene quantification method by HPLC. Parameters include the retention time (R_T), test range (µg), coefficient of determination (R^2), limit of detection (LOD, µg), limit of quantification (LOQ, µg), as well as accuracy and precision for the applied method. Values are a result of 3 replicate analysis for reference solutions prepared in the laboratory.

Compound	R_T (min)	*Range (µg)*	R^2	*LOD (µg)*	*LOQ (µg)*	*Accuracy (%)*	*Precision (%)*
Anthracene	15,3	10 – 200	0.99	6.21	18.85	97.3 – 102.7	14
Phenanthrene	14,5	10 – 200	0.99	4.28	13.9	98.1 – 103.5	10

The results (Figure 4.6) showed the success of the selection method used in AEx, given that only 3 strains presented low degradation activity – BBC|567, BBC|584 and BBC|593. These could correspond to slow growing strains, which present the necessary metabolic pathways, however the assay timeframe did not allow for clear growth and PAH metabolization. Also, they could represent a fraction of co-evolving strains which were assisting other microbial strains to survive the AEx, conditions, with lower fitness when grown alone.

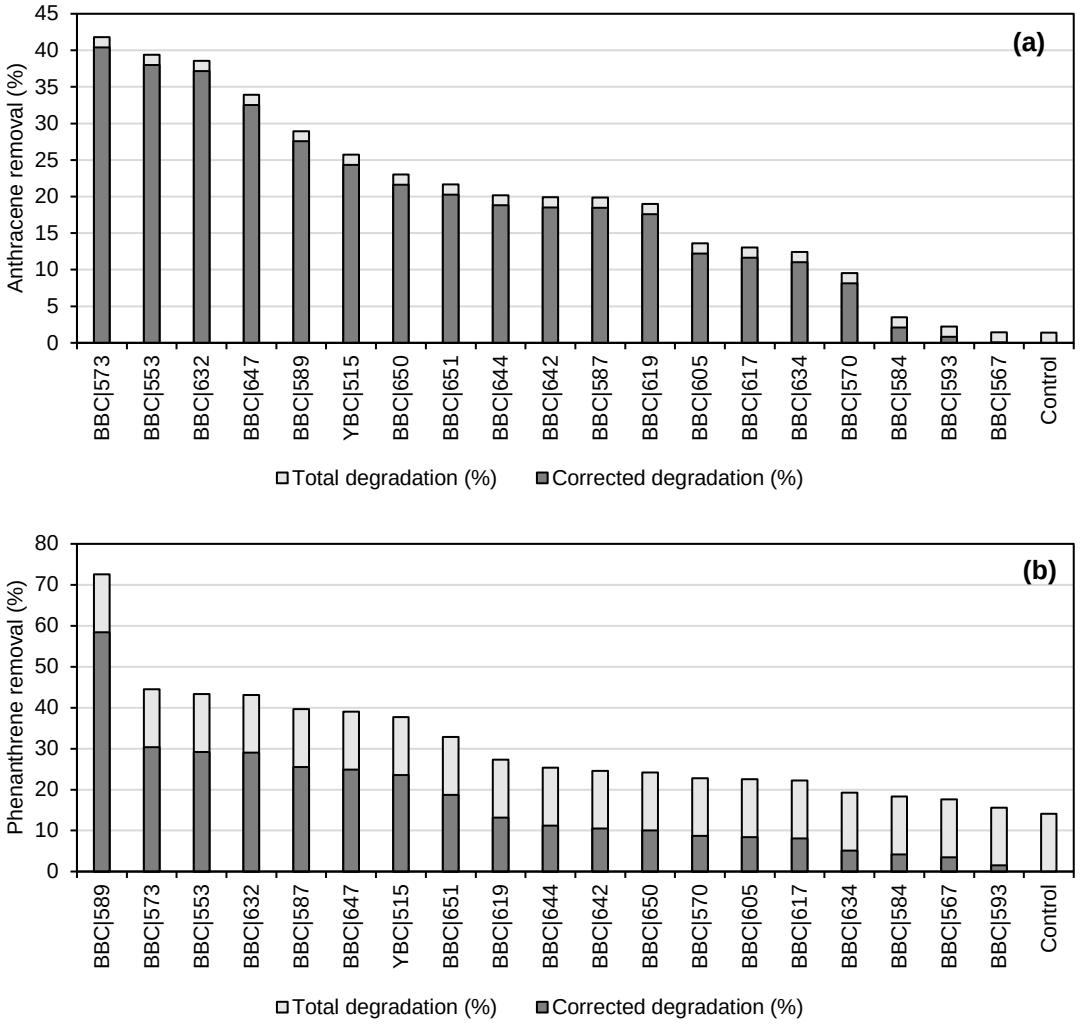


Figure 4.6 – Biodegradation 7-days assays in mineral medium M9 supplemented with different PAH's with selected strains. **(a)** with anthracene as sole carbon source; **(b)** with phenanthrene as sole carbon source. The corrected degradation (dark grey bar) was determined by subtracting the degradation value in the negative control for each substrate.

The strain BBC|520 was also tested, even though it was contained in the same fingerprinting cluster (data not shown) and genomically indistinguishable from representative strain BBC|573. As expected, similar degradation results were obtained, for both anthracene and phenanthrene, giving additional support for the goodness of the PCR-fingerprinting approach used for selection of strains from AEx.

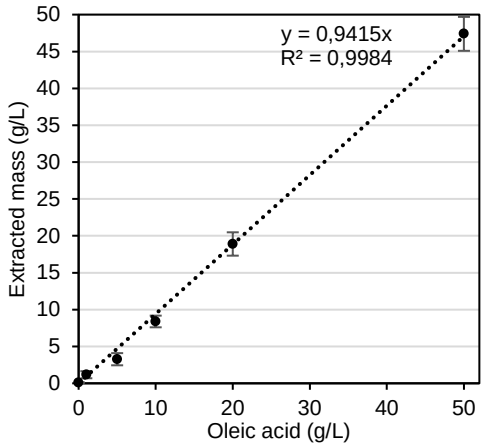
From these PAH degradation assays, some representative strains stand out as good degraders for both tested PAH, as BBC|553, BBC|573, BBC|589 and BB|632 that displayed average top results for both recalcitrant compounds given the test conditions. The strains BBC|573 and BBC|589 belong to genera with known PAH catabolism (Table 4.1), *Pseudomonas* and *Paraburkholderia*, while BBC|632, was genomically close to BBC|619, a *Pseudomonas* sp. strain, as showed in Figure 3.7f. Chowdhury and colleagues (2017) followed the biodegradation of anthracene and phenanthrene by a *Pseudomonas aeruginosa* strain and a *Stenotrophomonas maltophilia* strain, that reached 90% removal of anthracene after 20 and 48 days, while for phenanthrene 56 and 52 days were required, respectively. These strains showed interesting results, even though PAH removal was indirectly followed by a redox indicator, 2,6-dichlorophenolindophenol (DCPIP) while requiring quite long time to reach similar degradation level of strain BBC|589. On the other hand, *Bacillus cereus* strain S13 (Bibi *et al*., 2018) could remove 82% of anthracene after 120 h growth, with an initial concentration of 1 g/L of PAH. As for phenanthrene, Weissenfels and colleagues (1990) analysed three bacterial strains (identified as *Pseudomonas paucimobilis*, *Pseudomonas vesicularis* and *Alcaligenes denitrificans*), and reached a maximum PAH degradation rate of 0.1 mg/mL.day^{-1}. The discrepancy of microbial efficiencies described in the literature towards PAH bioremediation are derived from different experimental set-ups, quantification discrepancies (direct and indirect methods), and use of single bacteria versus consortia. The removal percentages of anthracene and phenanthrene achieved by adaptively evolved BBC strains in 7-days assays were quite good, considering the absence of pre-adaptation and relatively low cell density of the inoculum, the stringency of M9 mineral medium, and the reliability of determination method. In fact, the effective PAH quantification, using a total sample extraction, guarantees a real PAH metabolization by bacterial action, as demanded for a functional bioaugmentation strategy.

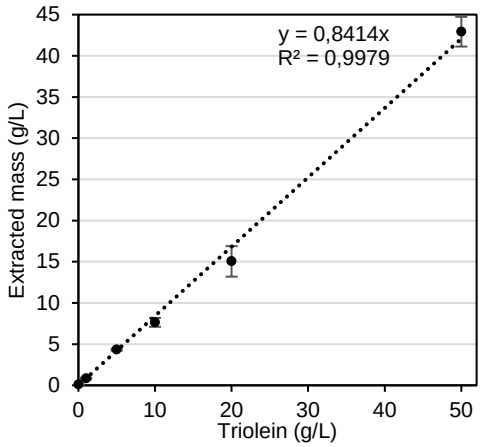
4.3.4 FOG biodegradation assays

The EPA method 1664 for FOG and Total Hydrocarbon quantification was frequently used in this work. A thorough analysis showed variability issues with this method, presenting an

estimated accuracy of 60% to 90%, depending on the laboratory (USEPA, 2010). For this reason, a validation step was conducted to determine the quantification limits obtained in our laboratory. The gravimetrical procedure is routinely applied in laboratories for FOG quantification in wastewater analysis around the world (USEPA, 2010). In our analysis, two pure laboratory substrates (oleic acid and triolein, from Fluka) and wastewater residue from a grease separator (top layer of fat and grease) were applied to test the extraction and quantification by the international method. The results in Figure 4.7 show the linear relation obtained for the three tested substrates, with values of the coefficient of determination (R^2) above 99% for all.

In Table 4.5 are presented the validation parameters derived from the linear regressions presented in Figure 4.7. The quantification method performed quite well for both pure substrate and the wastewater residue. In all cases, the calculated LOD and LOQ were adjusted for the degradation assays, with range to at least 50 g/L of extractable FOG. The accuracy obtained was quite high, which can be easily explained using pure substrates (in the case of oleic acid and triolein) and decanted oil, without visible contaminants to hinder the extraction procedure.





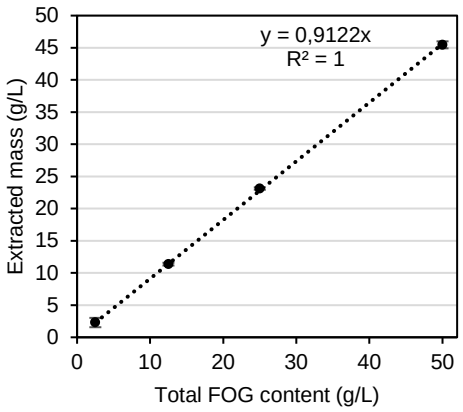


Figure 4.7 – Calibration curves with linear regression for oleic acid, triolein and FOG residues. In the linear regression, the points represent a mean of the measures with the respective standard deviation. The linear regression equation and the coefficient of determination are also depicted.

Table 4.5 – Validation parameters for the linear regression for the quantification of oleic acid, triolein and FOG residues. Parameters include the test range (g/L), coefficient of determination (R^2), limit of detection (LOD, µg/L), limit of quantification (LOQ, g/L), as well as accuracy and precision for the applied method. Values are a result of at least 3 replicates.

Compound	*Range (g/L)*	*R^2*	*LOD (g/L)*	*LOQ (g/L)*	*Accuracy (%)*	*Precision (%)*
Oleic Acid	10 - 50	0.99	0.23	0.6	104.9 – 113.6	7.6
Triolein	10 - 50	0.99	0.31	0.9	91.2 – 101.4	6.6
FOG	2.5 - 50	0.99	0.74	2.2	96.6 – 101.3	1.3

A fundamental step for the consistency of the method was the centrifugation one, prior to collect the organic phase (*n*-hexane). This step was added after several trials with very high variable results for reference solutions, following the EPA recommendations (USEPA, 2010). The development of a specific method, with low variability and high precision was fundamental for the subsequent steps in the biodegradation assays to evaluate all the selected strains. A biodegradation assay was conducted in M9 medium with 20 g/L of wastewater FOG residue, after 3- and 5-days growth at 28 °C in continuous shaking, to compare the activity of strains, selected from different approaches, with substrate from real grease separator (Figure 4.8). The initial inocula were kept around 10^8 to 10^9 CFU/mL, determined by the MPN method, to mimic previously assayed conditions. The set of tested strains included the previously selected strains *Aeromonas* sp. BBC|043 and *Staphylococcus* sp. BBC|148 (in Chapter 2), the 19 selected strains from the AEx described in Chapter 3, and two commercial products for bioaugmentation of FOG contaminated wastewater from company BioTask, for benchmark.

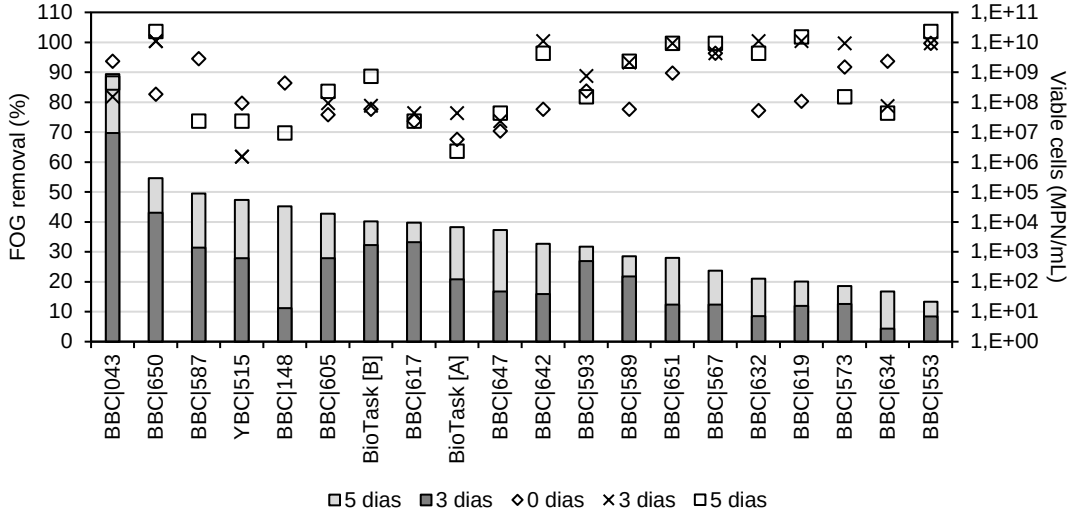


Figure 4.8 – Biodegradation assay in mineral medium M9 supplemented with real FOG residue as sole carbon source, with the strains selected from direct isolation method (BBC|043 and BBC|148) and the selected representative strains from AEx. Symbols refer to the most probable number (MPN) determined for the initial inoculum (◊), after 3- (×) and 5-days (□) incubation.

As shown in Figure 4.8, the best results were obtained with BBC|043, BBC|650, BBC|587 and YBC|515, strains from different isolation strategies, with FOG removal of 69%, 43%, 31% and 28%, respectively, after 5- days assay. Even though the commercial products presented some activity, eight strains achieved at least the same FOG removal efficiency. These were promising results, given the low nutritional medium used and the relatively low temperature, without prior inoculum activation of the necessary metabolic pathways. Some authors have used cooking oil, beef tallow and palm oil as substrate (Shon *et al*., 2002; Matsumiya *et al*., 2007; Sarmurzina *et al*., 2013; Emeer *et al*., 2014; Gao *et al*., 2019), however these are not a real wastewater residue, contaminated with all cleaning agents (detergents, surfactants, disinfectants) used in industrial kitchens. Wakelin and Forster (1997) used a strain of *Acinetobacter* sp. for microbial treatment of wastewater from fast-food restaurant to reduce FOG content, for 8-days treatment after inoculation with 10^7 CFU/mL, degrading 60–65% of fatty materials, but starting from 8 g/L, an initial FOG content much lower than the 20 g/L as in our biodegradation assay. Also, the initial inoculum used in the present work is comparable to recommendations for bioaugmentation procedure with commercial products for similar wastewater treatment, with very small differences found during the assay. Overall, the viability was slightly increased during the assay, or at least did not reduce over 5-days (Figure 4.8), indicating microbial biological activity during the process.

4.3.5 Microbial risk assessment

The objective of biotechnological bioaugmentation strategies is to use selected microorganisms to promote the degradation of a specific or overall pollution content of the water body or wastewater. The selected microorganisms must not present a risk for the environment and specially for human health. Furthermore, the selection of a hazardous strain could present a risk for subsequent treatment systems or for further water reuse. However, given the high level of pollution where these processes take place, as a WWTP, already considered a biologically dangerous workplace, there are less strict rules regarding the use and application of microorganisms. However, for human manipulation, microbial growth conditions and regulatory approval, the use of Generally Regarded As Safe (GRAS) microorganisms is recommended, and, if not, a microorganism not listed in Risk Group II or above, according to WHO and the Portuguese national law. In the present work, a putative identification by partial sequencing of reference gene for bacteria (16S rRNA gene) and yeasts (26S rRNA gene) was applied to the selected strains, for microbial risk assessment. Given the origin of these strains, from natural inocula, obtained by both direct and selective

isolation, without genetic engineering modifications, the taxonomic affiliation determines the biological risk for future bioremediation applications. As seen in Table 4.6, all strains could be associated to known species, with previously described lipolytic or hydrocarbon degradative genes. The majority belong to genera of gram negative Proteobacteria (*Acinetobacter*, *Aeromonas*, *Brevundimonas*, *Ochrobactrum*, *Paraburkholderia*, *Pigmentiphaga*, and *Pseudomonas*), few to genera of gram positive Firmicutes (*Bacillus* and *Staphylococcus*) and also to the *Yarrowia lipolitica* yeast species. As expected, there is a quite high diversity in the BBC collection, derived from the two isolation strategies used, previously described in Chapter II and Chapter III. These results are in accordance with the NGS approach on consortium dynamics, in Chapter III, detecting all these genera in the AEx. In the case of bacteria, the genera which stand out to differentiate between AEx were *Burkholderia*, *Pseudomonas* and *Acinetobacter* (Figure 3.9), to which belong most of the isolated strains that were selected. For fungi, the *Yarrowia* yeast genus was also identified as a major element to discriminate between AEx, as showed in Figure 3.10.

Table 4.6 – Partial *BLAST* results concerning taxonomically relevant genes: 16S rRNA gene for bacteria; 26S rRNA gene for yeasts. [a]*nt* stands for nucleotides. [b] closest relative hit retrieved from BLAST with reference material in NCBI database, presenting the sequence reference number in brackets.

Representative strain	***Length (nt)[a]***	***Closest relative [b]***	***Identity (%)***
BBC|043	877	*Aeromonas media* strain ATCC 33907 (NR_119041.1)	99
BBC|148	721	*Staphylococcus cohnii* strain GH 137 (NR_036902.1)	99
BBC|553	999	*Ochrobactrum anthropi* ATCC 49188 (MH281752.1)	99
BBC|567	1221	*Pseudomonas nitritireducens* strain WZBFD3-5A2 (NR_133020.1)	99
BBC|573	898	*Pseudomonas knackmussii* B13 (NR_121733.1)	98
BBC|584	995	*Ochrobactrum haematophilum* strain CCUG 38531 (NR_042588.1)	98
BBC|587	1158	*Brevundimonas diminuta* strain NBRC 12697 (NR_113602.1)	99
BBC|589	1173	*Paraburkholderia fungorum* strain LMG 16225 (NR_025058.1)	98
BBC|593	1114	*Pigmentiphaga kullae* strain K24 (NR_025112.1)	99
BBC|605	930	*Acinetobacter proteolyticus* strain NIPH 809 (NR_148846.1)	97
BBC|617	474	*Sphingobacterium multivorum* strain IAM 14316 (NR_040953.1)	98
BBC|619	1181	*Pseudomonas nitritireducens* strain WZBFD3-5A2 (NR_133020.1)	99
BBC|634	503	*Sphingobacterium multivorum* strain NRRL B-14861 (EU075197.1)	98
BBC|647	921	*Sphingobacterium multivorum* strain HMF3876 (KT983989.1)	99
BBC|650	1146	*Pseudomonas nitritireducens* strain WZBFD3-5A2 (NR_133020.1)	99
YBC|515	503	*Yarrowia lipolytica* strain VIT-ASN04 (KX258654.1)	99

The species encompassing the BBC strains have been previously described with lipolytic activity and potential use in bioaugmentation. Tzirita (2012) has discussed the beneficial use of isolates from *Bacillus* and *Pseudomonas* genera for bioaugmentation in oily wastewater, in association with commercial bioaugmentation products. Cammarota and Freire (2006) also reported on isolates from several *Acinetobacter* spp. and *Pseudomonas* spp. with capacity to reduce lipidic content, while several authors reported *Staphylococcus* spp., *Aeromonas* spp. and *Burkholderia* spp. for biodegradation of oily residues (Matsumiya *et al*., 2007; Prasad and Manjunath, 2011; Sarmurzina *et al*., 2013). Regarding PAH biodegradation, different bacterial species have been reported including *Achromobacter xylosoxidans, Acinetobacter calcoaceticus, Aeromonas* spp., *Alcaligenes denitrificans, A. faecalis, A. denitrificans, Arthrobacter polychromogenes, Bacillus cereus, Beijerinckia* spp., *Burkholderia cepacia, Corynebacterium venale, Cyclotrophicus* spp., *Flavobacterium* spp., *Micrococcus* spp., *Moraxella* spp., *Mycobacterium* spp., *Nocardia* spp., *Ochrobactrum anthropi*, *Pseudomonas maltophilia, P. paucimobilis, P. putida, P. testosteroni, P. vesicularis, Rhodococcus erythopolis, Sphingomonas agrestis, S. elodea, S. yanoikuyae, Staphylococcus saprophyticus, Stenotrophomonas maltophilia*, *Streptomyces* spp., *Vibrio* spp. (Samanta *et al*., 2002; Chaillan *et al*., 2004; Chadhain *et al*., 2006; Fang *et al*., 2006; Tanase *et al*., 2013; Kumari *et al*., 2018b). Black yeasts from *Cladophialophora immunda* and *Exophiala mesophila* with hydrocarbonoclastic activity were also described as potential bioremediation agents (Blasi *et al*., 2016). The non-conventional yeast *Yarrowia lipolytica* has been thoroughly studied, is considered a model for n-alkanes degradation studies and labelled GRAS species, considered an interesting candidate for future applications (Barth, 2013).

4.4 Conclusions

To conclude, two protocols were developed and evaluated, for both PAH and FOG quantification, for synthetic and real wastewater residues. Although there are validated methods routinely used for such purpose, each laboratory should confirm the quantification parameters (linearity, accuracy, precision, limits of detection and quantification). The obtained results showed the capacity limits for these methods to evaluate previously selected strains for PAH and lipids degradation.

In an initial study for growth capacity at microplate level, most selected strains were able to growth with the tested pollutants (anthracene, phenanthrene, tristearin and lubricant oil) as sole carbon source, confirming the screening process. Then, the effective biodegradation capacity was assessed in flask assays, using each strain in the presence of different

compounds. For PAH degradation, four strains presented the best results, namely *Ochrobactrum* sp. BBC|553, *Pseudomonas* sp. BBC|573, *Paraburkholderia* sp. BBC|589 and *Pseudomonas* sp. BB|632, for anthracene and phenanthrene removal after 5-days assay. The strains reached 37–40% removal efficiency for anthracene and 29–58% for phenanthrene, encouraging results comparable to other authors. Considering the FOG residues, both purified substrates and real wastewater residues were tested, with impressive results for *Aeromonas* sp. BBC|043, *Pseudomonas* sp. BBC|650 and *Brevundimonas* sp. BBC|587. They achieved 28–69% FOG removal after 5-days assay, in mineral media supplemented with 20 g/L of lipidic content derived from a grease separator wastewater residue. Several strains were capable to exceed the capacity for commercial products activity, specially designed for FOG residues, in comparable situations. All the strains were associated to genera with known biodegradation ability for PAH and/or FOG, being known for some of these genera the underlying metabolic pathways. Nevertheless, these strains could present novel genes of catabolic enzymes, transporters or even activation systems (Table 4.3), established during the evolution process, although further analysis should be conducted.

The screening and selection methodology allowed to select strains with higher biodegradation capacity than even available commercial products, to target specific pollutants, as PAH's, and undefined pollution, as FOG residue. These strains were obtained both from the direct screening of WWTP samples (*Aeromonas* sp. BBC|043 and *Staphylococcus* sp. BBC|148) and from the adaptative evolution approach (the remainder). Moreover, the AEx were determinant to isolate microorganisms in the selective solid medium, as was previously discussed in Chapter III.

The putative molecular identification showed the selection of various taxa, as *Pseudomonas* and *Aeromonas* from bacteria to *Yarrowia* from yeasts, genera encompassing degradative species, with known impact on the environment. Given the origin source of these strains, from WWTP, and from AEx, they should be capable to proliferate in these dynamic and aggressive environments, with toxic compounds, derived from man-made pollutants. The capacity to persist and occupy a significant presence in any wastewater is a central feature for a successful bioremediation process in real scale applications. These strains have great features for the design of new bioaugmentation products, to promote lipids or hydrocarbon degradation, for both *in situ* and *ex situ* approaches.

Chapter V – Future Perspectives

5.1 Focus on *Innovation*

The concept of *Innovation* is associated to new ideas, methods, devices, with the introduction of something new. That is, without a question, one of humans most unstoppable force: the ability to change, move, choose differently, with more responsibility. In a time facing global problems, as climate change, the rise of sea-level, pollution and contamination problems found in our landscape, our oceans and our atmosphere, it is fundamental to innovate to find novel technologies and solutions to invert these processes.

Biotechnology has been known for specific problem-solving solutions, as an industrial way to produce biological relevant compounds, with antibiotics, hormones, bioplastics, or enzymes as examples. The bioremediation field, even though integrated into biotechnology, has been a slower little brother, with lower investment and scarce public appearance. It was undoubtedly separated from the wastewater treatment engineering school, which focus on the infrastructural and mechanical side of WWTP, with little regard for the microbiological component. The separation has put bioremediation in a grey area, with few practical examples in real-scale and very low acceptance by common WWTP professionals. There is a need to demonstrate the potential benefits and applicability of bioremediation as an efficient tool to improve biological treatment of effluents.

The present work focus on the development of bioremediation products, setting up the initial stages for a Research and Development (R&D) Department within the company BioTask, for design and validation of novel products. The isolation of microorganisms for such goal presents an innovative way of valorisation of our national biological potential, developing our own products, involving the national scientific institutions for microbiology and biotechnology. The growing concern with environmental causes, associated to the continuous production over the necessary pollution treatment, will lead to an increasing need for such alternatives. We rely on the naturally occurring microorganisms to catabolize our pollution, and we will need to strengthen this activity. This work presented the bases for new product design development, in Portugal, from natural samples, focusing on two recognised problematic areas for both WWTP and industries. There are different possibilities for bioremediation applications, from house-hold products, to industrial specific inoculum design, to efficiently introduce degradative strains into the pipe-systems, as an example. On the other hand, the methodologies will allow future scale-up assays to demonstrate the capacity of such approach.

The environmental area requires a strict and vigilant law imposing force, or a strong public awareness over pollution impact in the environment. One of the current challenges is to create

a sustainable environment, without critically affecting the industrial production. To achieve this, more efficient treatment systems for wastewater disposure or reuse are required, as well as more economical ones. Bioremediation is associated with both concepts (sustainability and economic feasibility), presenting alternative solutions to promote a healthier environment, accelerating the natural biological cycle in an intelligent and focused manner. Unfortunately, Portugal still has many problems to overcome, including in the legislation department. There is still limited legislation over soil contamination and rehabilitation requirements, while the air pollution control is only required for specific industries. Water is the most regulated area, especially for consumable water, while the wastewater treatment regulations concern mostly unspecified pollution, as organic matter, chemical oxygen demand, or nutrient levels. In addition to national law, municipalities and industries have started to impose quality standards higher than the demands, in response to the increasing public awareness.

The current research points to a low diversity amongst strains incorporated into commercial products for bioaugmentation, usually containing *Bacillus* spp. and/or *Pseudomonas* spp.. Much of the research focuses on the naturally occurring strains (bacteria and fungi) with quite interesting results for biodegradation, especially at laboratory level, and some at pilot scale. There is little doubt that the selection of different strains, from other genera, present a novelty route for products development, to improve biological degradation of pollutants. Also, the selection of naturally occurring microorganisms, from WWTP in our environment, will lead to strains much more related with the original microbiome. As for the strains selected from the adaptive evolution experiments (AEx), they represent interesting populations which were maintained over 100 cycles, for a 2-year period, in quite difficulty physiological conditions (limiting nutrients and carbon source). The resilience demonstrated by these strains represents an acquired advantage over other strains, to colonize and influence such a dynamic and fast evolving system as a biological WWTP microbiome.

5.2 Research topics suitable for follow-up

The BBC culture collection provides a potential microbial source of new degradative metabolic pathways, enzymes, biosurfactants or other by-products. Given the isolation source and the isolation methods applied, there could be other interesting activities amongst these isolates which were not analysed and fail to exceed, given the initial main objectives. Similar pollutant compounds should be further analysed, as phenolic, phthalic or chlorinated compounds (with similar metabolic routes for PAH catabolism). Also, these isolates may present specific hydrolase activity, a key enzymatic step for different substrates assimilation,

as amylase, sucrase, or proteases. They could represent low cost producers, able to convert wastewater residues into valuable biotechnological products with industrial applications.

The increasing availability for genomic approaches presents a new tool to further increase the knowledge for promising microbial strains. A whole-genome sequencing could unravel hidden alternative enzymatic clusters, new metabolic pathways, improved catabolism from isolates obtained from such specific laboratory set-up. These are possibilities derived from the increasing development of sequencing methodologies, only limited by our own capacity to analyse the obtained data in a correct, proof-reading and up-to-date manner.

Furthermore, other pollutants represent challenging obstacles for biological processes, such as hormones, antibiotics, and pharmaceuticals. These could represent areas of innovative products specially designed to attenuate the presence of recalcitrant pollutants. Microbial consortia presenting the necessary metabolic pathways for hormone catabolism could bridge the gap between traditional WWTPs to create an efficient biological system focusing on hormone removal, as an example.

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Supplementary Information

Table S1 – Oligonucleotide primers used in the present work for different applications. Primers are discriminated by the target gene, nucleotide sequence and application.

Primer	Genetic target	DNA sequence (5' - 3')	Application	Reference
PH	16S rRNA gene	AAGGAGGTGATCCAGCCGCA	Single primer fingerprinting	Massol-Deya *et al.*,1995 Edwards *et al.*, 1989
csM13	M13 minisatellite	GAGGGTGGCGGTTCT		Huey and Hall, 1989
$[GTG]_5$	Genomic microsatellite	GTGGTGGTGGTGGTG		Schafer *et al.*, 1988
pA	16S rRNA gene	AGAGTTTGATCCTGGCTCAG	Gene sequencing	Lane, 1991
1392r		ACGGGCGGTGTGTRC		Edwards *et al.*, 1989
NL1	26S rRNA gene	GCATATCAATAAGCGGAGGAAAAG		O' Donnell, 1993
NL4		GGTCCGTGTTTCAAGACGG		Guého *et al.*, 1989
PAH-RHD-GNf	Ring-hydroxylating dioxygenase gene in Gram negative bacteria	GAGATGCATACCACGTKGGTTGGA	Functional gene amplification	Cébron *et al.*, 2008
PAH-RHD-GNr		AGCTGTTGTTCGGGAAGAYWGTGCMGTT		
PAH-RHD-GPf	Ring-hydroxylating dioxygenase gene in Gram positive bacteria	CGG CGCCGACAA YTTYGTNGG		
PAH-RHD-GPr		GGGGAACACGGTGCCRTGDATRAA		
C12Of	Catechol 1,2 dioxygenase gene	GCCAACGTCGACGTCTGGCA		Sei *et al.*, 1999
C12Or		CGCCTTCAAAGTTGATCTGCGTGGT		
C23Of	Catechol 2,3 dioxygenase gene	AAGAGGCATGGGGGCGCACCGGTTCGATCA		
C23Or		CCAGCAAACACCTCGTTGCGGTTGCC		
sgp319f	Salicylate 5 hydroxylase gene	TGCCCCTAYCAYCARTGG		Izmalkova *et al.*, 2013
1238r		CGCCAGTABTBGTACATGCC		

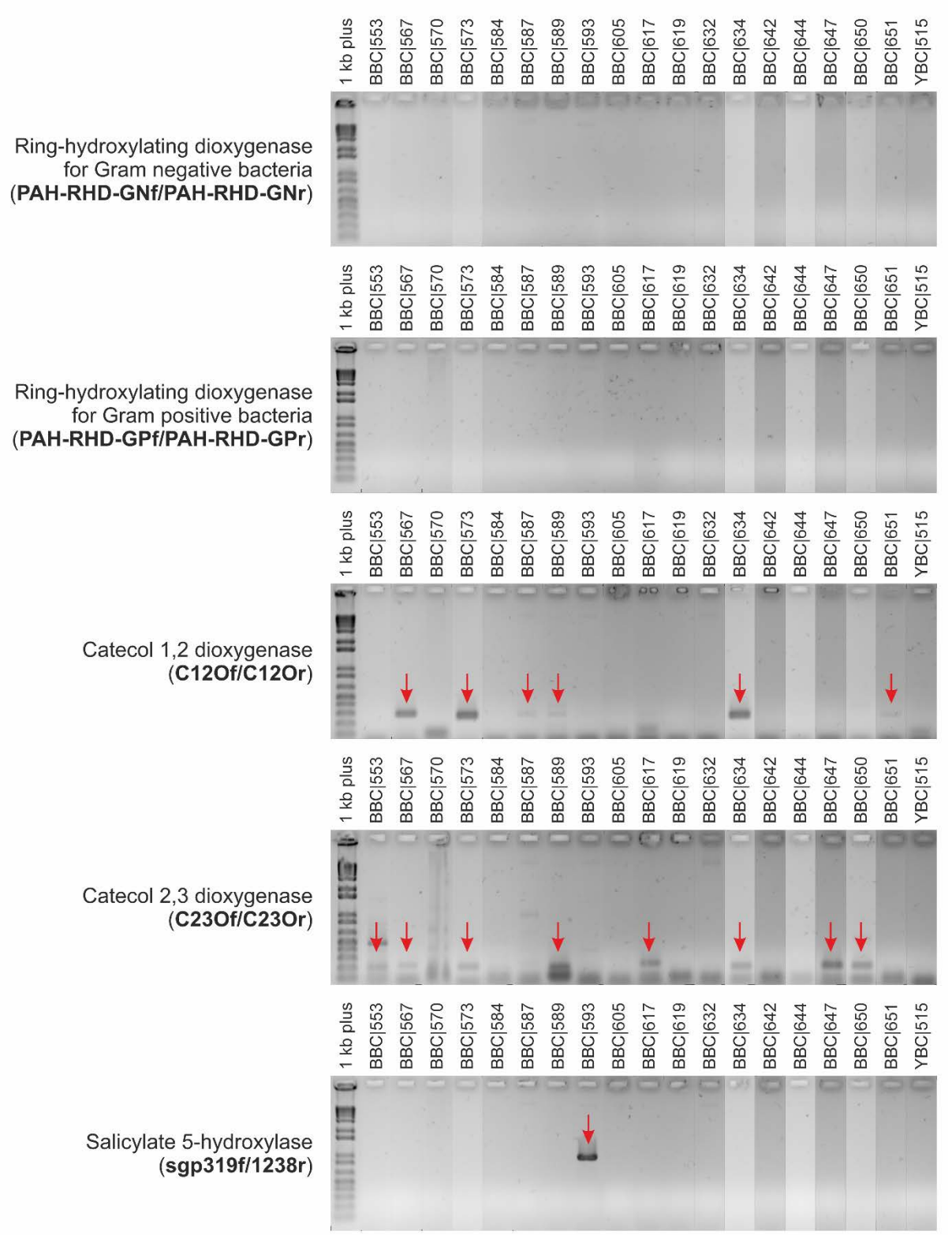

Figure S1 – PCR amplification results for the search of functional genes. Each run represents different gene targets for PAH catabolism: ring-hydroxylating dioxygenases for Gram positive bacteria, and for Gram negative; catechol 1,2 dioxygenase; catechol 2,3 dioxygenase; salicylate 5-hydroxylase. The amplification fragments presenting the expected size were considered positive results and pinpointed by the red arrows. In bold are the primers used in each PCR amplification.